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Genetic and Biochemical Studies of the Biosynthesis and Attachment of D-Desosamine, the Deoxy Sugar Component of Macrolide Antibiotics Produced by *Streptomyces venezuelae*

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by

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Dedication

To Yuri

Acknowledgements

I have been very lucky to be working in the Liu lab for all these years. When I was choosing the lab to work in back in Minnesota, a friend of mine advised me to join Ben's (Hung-wen Liu's) lab. He said that I may never be the best student in this lab, but I will learn from the best. He was right. Working with Ben and his team was certainly the best way to get the maximum out of my time in the Graduate School. I would like to thank Ben for his supervision and support. Not only did I learn from him a great deal about biochemistry in general and enzymes in particular, but also he was very supportive during my academic endeavors.

I started out with Ben in Minnesota, and as a new student drew on from a lot of "old" people in the group, or as they call them here now, "former people". I have learnt most of the stuff that I know about molecular biology and working with *Streptomyces* from my mentor, Lishan Zhao, the farther of the "Desosamine Project". Lishan is a great teacher, an extremely productive researcher, and a very nice person to work with. I also turned very often to Raymond (Huawei) Chen, or "wise Raymond", for the information on protein chemistry. In fact, I still bug Lishan and Raymond every once in a while for some valuable advice.

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Moving to Austin gave me a chance to get much closer to the group members who also moved. Hiroshi Yamase, who was my desk neighbor at UT for couple of years, does not talk much, which is sometimes a good thing for the person who sits next to you. However, he was always there when I needed help, either in chemistry or to move heavy objects. Hiroshi was a pleasant companion

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filled weekends together quilting, baking cookies, and listening to Yuri's "knowledgeable comments" on quilting. From Vidusha I also learned to love and, more importantly, cook Indian food. Alex Wong has the best sense of humor and I did and will always enjoy his company. He is also a very broadely knowledgeable person, but is particularly an expert in cooking. Despite the fiery exterior, Alex is a very approachable and an extremely selfless individual; goodness knows how many times he helped me with my chemistry, writing, cat caring and multiple other tasks. Now that he is a father, there will be one more reason to request his expert opinion. The life in the Liu lab would not have been the same without Sophie's-mom-to-be, HuA Zhang. Hua is our molecular biology specialist. She also makes the best dumplings, is always cheerful, and is an extremely enthusiastic sports fan. She throws the greatest Chinese New Year parties and improves English words in the funniest way. Our "dreaming vacation" with Alex, Beth, Hua, Vidusha, and Yuri was unforgettable.

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Genetic and Biochemical Studies of the Biosynthesis and Attachment of D-Desosamine, the Deoxy Sugar Component of Macrolide Antibiotics Produced by *Streptomyces venezuelae*

Publication No. _____

Svetlana Alekseyevna Borisova, Ph.D.

The University of Texas at Austin, 2004

Supervisor: Hung-wen Liu

Macrolide antibiotics are clinically important drugs widely used to treat the infections caused by gram-positive bacteria. They consist of a macrolactone aglycone unit and one or more deoxy sugar component(s). The presence of an at least one deoxy sugar moiety in the structure of macrolides is essential for their antimicrobial activity. Modifications of the deoxy sugar substituent hold promise as a valuable approach towards generating new macrolide antibiotics with improved biological properties. The development of new antimicrobial drugs is essential to combat the growing problem of the pathogen resistance to the existing antibiotics.

This thesis describes a part of our ongoing effort to investigate the mechanistic details of the biosynthetic pathway to D-desosamine, an amino deoxy

sugar component of macrolide antibiotics methymycin, neomethymycin, pikromycin, and narbomycin produced by *Streptomyces venezuelae*. D-Desosamine also exists in many other clinically important macrolides, *e.g.* erythromycin, clarithromycin, and oleandomycin. In particular, the gene knockout technique was used to explore the functions of the *desI*, *desII*, and *desVIII* genes of the D-desosamine biosynthetic gene cluster. The analysis of the macrolides produced by the resulting *S. venezuelae* mutants has led to the revision of the originally proposed pathway to desosamine. A new mechanism for the C-4 deoxygenation step was also envisioned. Four new macrolides were isolated in these studies, which established the relaxed substrate specificity of the glycosyl-transferase DesVII involved in the coupling of TDP-sugar derivatives to the aglycone in the pathway.

To further explore the potential of DesVII to couple various sugar and aglycone substrates, *desVII* was expressed in *E. coli* and the recombinant DesVII protein was used to study the *in vitro* glycosyltransferase activity. Our results demonstrate that DesVII requires an additional protein component, DesVIII, to perform the catalysis, and the activity is optimal at pH 9. These conditions, unusual for the known glycosyl transfer reactions, may prove to be the general requirements for other macrolide glycosyltransferases. The preliminary study of the substrate specificity of the DesVII/DesVIII catalytic pair was conducted *in vitro* and showed the potential for their application to glycosylation in combinatorial biosynthesis.

The mechanism of the self-resistance in *S. venezuelae via* glycosylation-deglycosylation was also studied. The gene encoding the resistance glycosyltransferase DesG was identified in the genome of *S. venezuelae*. It was later expressed in *E. coli* and the DesG protein was purified. The substrate specificity of DesG was found to be relatively relaxed.

Overall, this study provides significant insights into the biosynthesis of macrolides by *Streptomyces*. It has also demonstrated the feasibility of preparing novel compounds using genetic manipulations of the biosynthetic machinery. The discovery of DesVIII as an essential component in the glycosyl transfer catalyzed by DesVII resulted in the first successful reconstitution of a macrolide glycosyltransferase activity *in vitro*. This work is an important step towards the creation of tailored macrolide antibiotics using "unnatural" sugar substrates and glycosyltransferases.

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Chapter 1: Introduction: Macrolide Antibiotics

1.1 MACROLIDE ANTIBIOTICS: THEIR ORIGIN AND MODE OF ACTION

The term "macrolide" was first introduced by Woodward[1] to define natural products which contain a 12- to 16-membered macrocyclic lactone ring. The macrolactone ring of a macrolide has been referred to as aglycone and is often decorated with one or more deoxy and/or amino sugars. While the original term "macrolide" is reserved for compounds containing no nitrogen atoms as part of the macrolactone ring, it has now been broadened to include semisynthetic nitrogen-containing antibiotics, such as azithromycin (**10**, Figure 1-1), and polyene and streptogramin antibiotics. A few representatives of macrolide antibiotics are shown in Figures 1-1 and 1-2. The vast majority of macrolide antibiotics are produced by various species of *Streptomyces*, but examples are known that are generated by other actinomycetes, such as *Micromonospora megalomicea* (megalomycins). There are also cases in which the same antibiotic is produced by different *Streptomyces* species. Macrolide antibiotics are preferentially active against gram-positive bacteria, such as *Staphylococcus* and *Streptococcus*. They possess only limited activity against gram-negative bacteria, mainly gram-negative cocci, such as *Neisseria gonorroheae* and *Haemophilus influenzae*.

The first macrolide antibiotic isolated and partially chemically characterized is pikromycin (**4**).[2] The isolation of methymycin (**1**) was reported in 1954.[3] It became the first macrolide for which the structure was determined.[4]

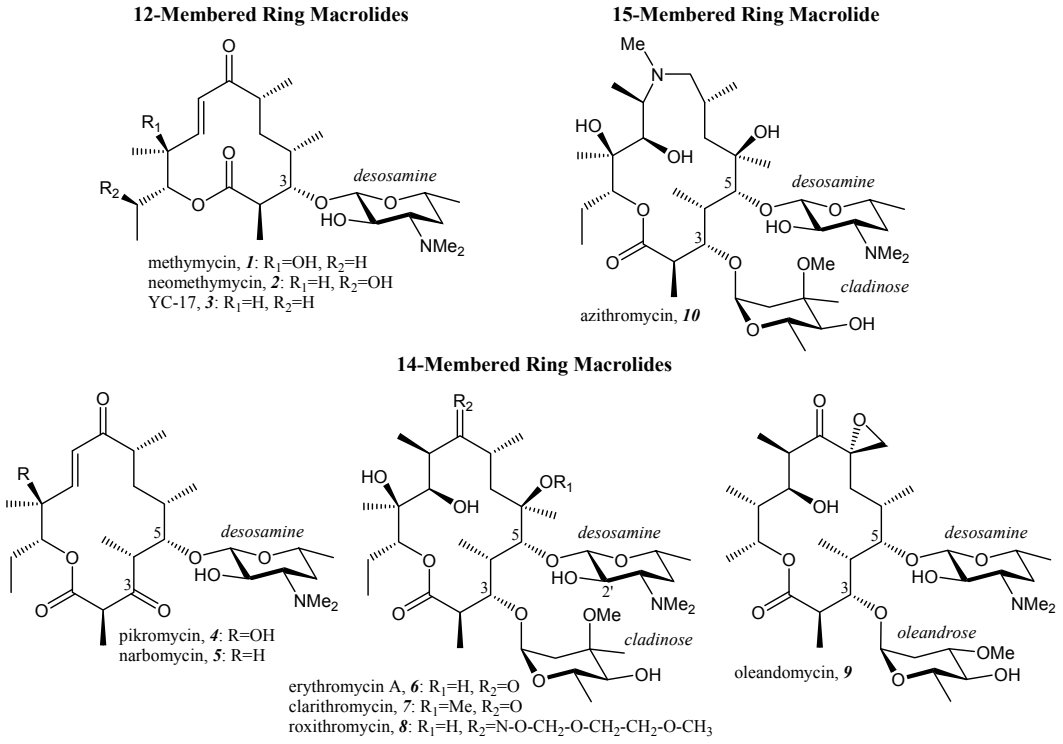

Figure 1-1: Examples of 12-, 14-, and 15-membered ring macrolides.

Macrolides are classified according to the size of the lactone ring. Methymycin (**1**), together with neomethymycin (**2**) and 10-deoxymethymycin (**3**, or YC-17), are the only naturally occurring 12-membered ring macrolides known, but they have not been used clinically. In contrast, the more prevalent 14- and 16-membered ring macrolides have been widely used and extensively studied. Of particular interest are the antibiotics of the erythromycin family. Erythromycin A (**6**) is the first clinically used macrolide which was launched as a drug in 1952.[5] It is still being used for treatment of bacterial infections because of its low cost, effectiveness, and low toxicity. A number of semisynthetic macrolides derived from erythromycin A with an improved stability and/or permeability have been

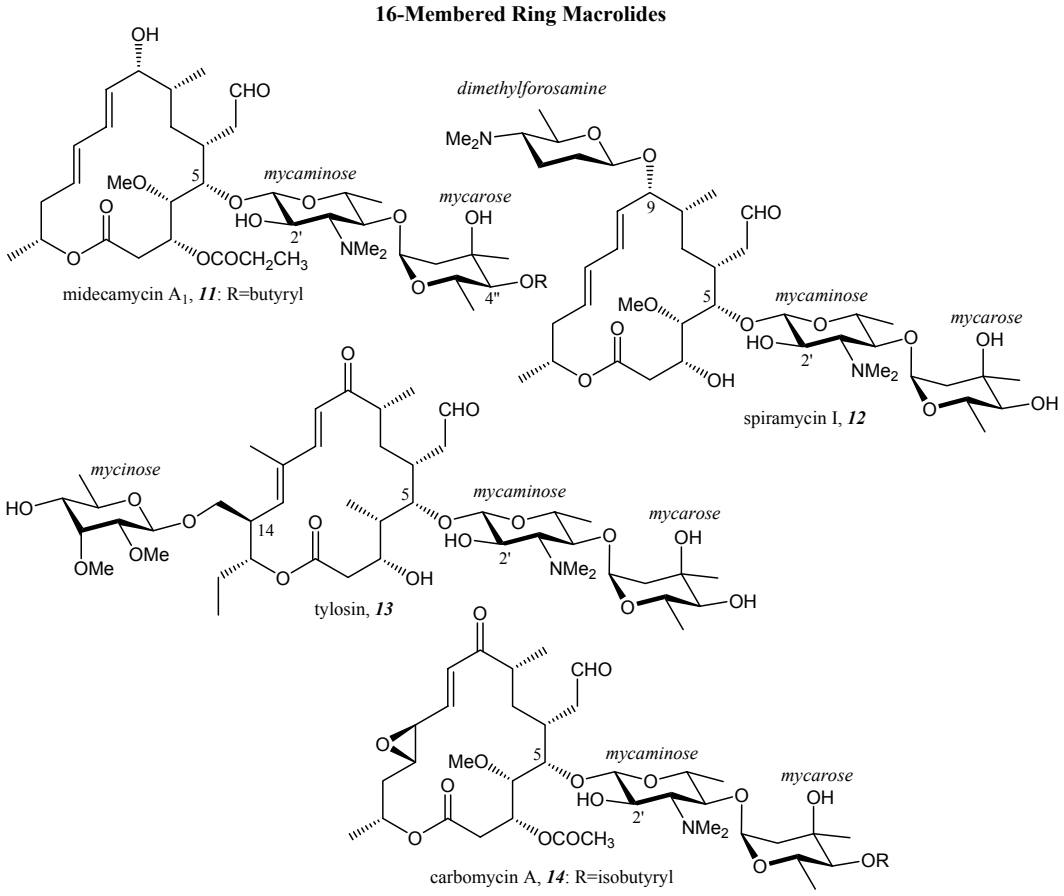

Figure 1-2: Examples of 16-memberd ring macrolide antibiotics.

developed, such as clarithromycin (**7**), roxithromycin (**8**), and the 15-membered ring azithromycin (**10**). Other clinically useful antibiotics include oleandomycin (**9**), midecamycin A_1 (**11**), and spiramycin (**12**). Tylosin (**13**) is widely used in the veterinary field.

Macrolide antibiotics selectively inhibit protein synthesis in prokaryotes. The observed selectivity is due to the differences in the eukaryotic and prokaryotic protein biosynthetic machineries. Macrolides are thought to block the

peptide exit tunnel, preventing the extension of the nascent peptide from the peptidyl transferase center. The general location of the macrolide binding site on the large ribosomal subunit 50S was initially determined using biochemical and genetic methods.[6-8]

Recently, results obtained from several crystal structure studies have provided more details of the macrolide-ribosome interactions.[9, 10] These include the complexes of the 50S ribosomal subunit of the eubacterium (*Deinococcus radiodurans*) with 14-membered erythromycin (**6**) and its analogs, clarithromycin (**7**) and roxithromycin (**8**).[9] The structures of the 50S ribosomal subunit of the archaeon (*Haloarcula marismortui*) with 15-membered azithromycin (**10**) and 16-membered spiramycin (**12**), tylosin (**13**), and carbomycin A (**14**) were also solved.[10] These structures are generally similar, and clearly reveal that the binding of macrolides to the polypeptide exit tunnel of the 50S subunit occurs predominantly through the contact with the 23S ribosomal RNA. In the case of the 16-membered macrolides, a covalent bond is formed between the aldehyde moiety and the 6-N of A2103 (*H. marismortui*, corresponds to A2062 in *Escherichia coli*).[10] These studies also showed that most of the binding interactions of macrolides with a ribosome arise from the contacts of their sugar substituents.[10] It should be noted that all seven macrolides used in the crystal studies contain a dimethylamino hexose (desosamine or mycaminose) at the C-5 position of each aglycone. The mycaminose is further glycosylated with mycarose in the 16-membered macrolides shown in Figure 1-2. Additional deoxy sugar residues are present at C-3 of erythromycin, clarithromycin, roxithromycin,

and azithromycin (cladinose), at C-9 of spiramycin (dimethylforosamine), and at C-14 of tylosin (mycinose). These sugars are important for the biological activity of each macrolide.[11-15] Particularly, the dimethylamino hexose moiety in each structure is indispensable for the antibacterial activity of the parent compound, although the neutral deoxy sugars, *e.g.* cladinose in erythromycin, are less essential.[16]

The conserved feature of the crystal structures of all seven macrolide-ribosome complexes is a hydrogen bonding interaction between the 2′-OH of desosamine, or that of mycaminose, and N-1 of A2058 (the *E. coli* numbering system).[9, 10] The complexes with *D. radiodurans* 23S rRNA have additional hydrogen bonds between 2′-OH of desosamine and the 6-NH_2 of A2058 and the 6-NH_2 of A2059.[9] Modifications of these rRNA residues have been implicated in resistance mechanisms (see below). Hydrophobic and hydrogen bonding interactions are also observed between the mycinose sugar of tylosin and the dimethylforosamine of spiramycin and ribosomal proteins L22 and L4, providing some structural evidence for an alternative resistance mechanism which has been suggested through mutations of these proteins.[10]

Interestingly, the X-ray crystal structures also show that the disaccharide moiety at C-5 of the 16-membered macrolides extends further into the exit tunnel toward the peptidyl transferase center than the monosaccharide appendage attached at the same position of other macrolides.[10] In fact, the length of this saccharide (di or mono) side chain directly correlates with the length of the short peptides generated by a ribosome in the presence of macrolides: the more the

sugar chain extends into the exit tunnel, the shorter the peptide that is produced by the ribosome.[10] Thus, the analysis of the crystal structure data has provided significant insight into not only the macrolides' mode of action, but also the resistance mechanisms developed by pathogens to neutralize the effects of these clinically useful drugs.

1.2 RESISTANCE TO MACROLIDE ANTIBIOTICS

After a new antibiotic is introduced, sooner or later the targeted organisms will develop a resistance to it. Recent efforts to combat the emergence of antibiotic-resistant bacteria mark a new level of humans' struggle with infectious diseases.[17, 18] It has been demonstrated that bacterial antibiotic resistance arises under the evolutionary pressure for survival, and once it appears in one strain, it can quickly spread to others.[5] This is because the resistance determinants (resistance genes) found in pathogens are predominantly located in plasmids, which are easily transferred from one bacterium to another, as well as between strains.[19]

Three major resistance mechanisms have been identified in pathogenic bacteria: (i) drug efflux from the cell, (ii) target modification, and (iii) inactivation of the antibiotic. Mechanisms (i) and (ii) are the most common strategies adapted by gram-positive pathogens to fight against macrolide antibiotics.[20] In fact, most antibiotic-producing species utilize one or more of these mechanisms to protect themselves from intoxication from their own metabolites. It has been suggested that antibiotic producing species might be the source of at least some of the resistance determinants found in the pathogens with an acquired resistance.[21]

1.2.1 Drug Efflux

Since the protein biosynthetic machinery is located in the cytoplasm, macrolides have to pass through the cell membrane in order to reach their target. A sufficient amount of the macrolide also needs to accumulate within the cell to achieve its effective concentration. Drug efflux can prevent the buildup of the intracellular concentration of the antibiotic, rendering the compound ineffective against its target.[22] Efflux pumps are found in all kinds of bacteria and are used to transport molecules through the membrane into and out of the cell. An active efflux using ATP-binding cassette (ABC) transporters has been reported as an effective means for self-resistance by the tylosin producer *Streptomyces fradiae*,[23, 24] the oleandomycin producer *Streptomyces antibioticus*,[25, 26] and a number of other antibiotic-producing organisms.[27] Several pathogens exploit efflux pumps to confer resistance to multiple antibacterials, including macrolides.[28] Recently, the resistance to 14- and 15-membered macrolides was found to be based on the ABC transporter system in *Staphylococcus aureus*.[29] An important macrolide resistance mechanism in a number of *Streptococcus pneumoniae* strains relies on the efflux pumps of a major facilitator superfamily (MFS).[30]

1.2.2 Target Modification

Perhaps the best-studied mechanism of macrolide resistance is the modification of drug binding sites in the ribosome. The mono- or dimethylation at the C-6 amino group of A2058 of 23S rRNA by a dedicated *N*-methyltransferase (Erm, erythromycin resistance methylase) is the "defense of choice" for a growing number of bacteria.[31] The first clinical isolates of

macrolide-resistant staphylococci, found shortly after the introduction of erythromycin, exhibited drug resistance based on such a post-translational modification of rRNA.[32] Interestingly, the macrolide antibiotics bind to the same target site on the ribosome as the structurally distinct antibiotics of lincosamide and streptogramin B families. Therefore, a site-specific methylation of rRNA usually confers cross-resistance to these three classes of drugs, and is often referred to as MLS_B resistance.

Examining the crystal structure of the 50S ribosomal subunit showed that the residue modified by Erm is located in close proximity to the peptidyl transferase loop.[33] This finding links the mutation, causing the macrolide resistance, to the action of the antibiotic as an inhibitor to block the early steps in protein synthesis. As mentioned before, the crystal structure of the complexes of the 50S ribosomal subunit with several macrolides also revealed that the 6-N of A2058 is involved in hydrogen bonding with the desosamine moiety of macrolides.[9]. Thus, dimethylation of the 6-N group not only adds a bulky substituents causing steric hindrance for the binding, but also prevents the formation of hydrogen bonds to the 2′-OH group of desosamine.[9]

The methylation of rRNA has also been shown to confer cellular self-protection to the producers of erythromycin (*Saccharopolyspora erythraea*),[34, 35] tylosin (*S. fradiae*),[36, 37] and carbomycin (*Streptomyces thermotolerans*).[38] The species employing this strategy usually exhibit MLS_B phenotype, *i.e.* they are resistant not only to the antibiotic they produce, but to other MLS_B drugs as well.

The MLS_B resistance can be constitutive if the production of active methyltransferase mRNA is independent of an inducer (antibiotic). It can also be inducible if activation of the *erm* mRNA (and therefore Erm production) occurs only in the presence of an antibiotic.[39] The latter type is more common for pathogens, in which the inducible resistance mechanism is turned on when an antibiotic of the MLS_B family stalls the translation of the mRNA fragment preceding the *erm* sequence. It is worth noting that the *erm* mRNA is normally present in the inactive conformation. The stalling of the translation of the upstream mRNA region by an antibiotic causes its rearrangement into an active conformation. This allows the translation of the *erm* gene into *N*-methyltransferase Erm.[40, 41]

There exists an alternative resistance mechanism by target modification in some pathogens, in which A2058 of the 23S rRNA is replaced by cytosine, guanosine, or uracil.[42] The neighbors of A2058 may also be mutated, *e.g.* G2057A and A2059G, in some cases. This strategy is often observed in species containing only a few copies (one or two) of 23S rRNA genes, such as *Helicobacter pyroli* and *Mycobacterium* species, whereas methylation of rRNA discussed earlier is the predominant strategy in bacteria carrying multiple 23S rRNA genes.[42] The crystal structures of the complexes of the 50S RNA subunit with different macrolides show that perturbations at these residues would impair the binding of antibiotics.[9, 10] The position corresponding to A2058 is occupied by guanosine in the rRNA of higher eukaryotes, and this could contribute to the selectivity of macrolides to bacterial ribosomes.[9]

Mutations of ribosomal proteins, most often L4 and L22, conferring erythromycin resistance are reported in *E. coli*,[31] and more recently, in clinical isolates of *S. pneumoniae*.[43] The proteins L4 and L22 constitute the constriction of the polypeptide exit tunnel. The amino acid residues mutated in the macrolide resistant strains are usually located in the highly conserved regions of the proteins. The role of these mutations in the macrolide resistance might be attributed to their indirect involvement in the local rearrangements of 23S rRNA as the result of amino acid sequence changes.[9] However, the interactions between mycinose of tylosin with L22 and dimethylforosamine of spiramycin with L4 are clearly discernible in crystal structures of the complexes of macrolides with the 50S ribosomal subunit and might be of some significance for drug binding.[10]

Recently, a new macrolide resistance mechanism was discovered in *E. coli*.[44] It was found that treatment with macrolides may induce the expression of specific short peptides of 4-6 amino acids. These peptides may then interact with the macrolide molecule bound to the ribosome, causing its displacement from the binding site – a "bottle brush" model.[45] A similar model is also possible in *S. aureus*,[46] but is yet to be confirmed in clinical isolates of pathogens. The details of this resistance mechanism remain elusive.

1.2.3 Inactivation of Antibiotic

A number of resistance mechanisms rely on enzyme-mediated inactivation of the drug. Unlike the two strategies described above, inactivation of the antibiotic confers resistance to structurally related compounds only. Several strains of *Enterobacteriaceae*, *e.g. E. coli* strains, reportedly produce

erythromycin esterase which catalyze lactone ring cleavage[47] and macrolide 2′-phosphotransferase I,[48] which target 14- but not 16-membered macrolides. On the other hand, both 14- and 16-membered macrolides can be phosphorylated by 2′-phosphotransferase II of *E. coli*.[49]

The presence of a macrolide glycosyltransferase activity in actinomycetes not producing macrolides has been reported in a number of cases and constitutes these species' resistance strategy. For example, *Streptomyces vendargensis* and *Streptomyces lividans*, which themselves do not produce any macrolides, are capable of inactivating erythromycin A by glycosylating the desosamine moiety at the 2'-OH position with a glucopyranose residue.[50-52]

Until recently, this type of mechanism was found only in non-pathogenic species (*e.g. Streptomyces*), or in bacteria not targeted by macrolides (*Enterobacteriaceae* species are not considered to be a good target for macrolides). However, pathogens employing macrolide inactivation as a defense mechanism were discovered recently. It now appears that the resistance of most pathogenic *Nocardia* species to macrolides is due to the antibiotic inactivation *via* 2′-phosphorylation, reduction of a formyl group of macrolactone (*e.g.* in midecamycin, **11**), deacylation (the removal of the acyl group from the 4′′ position of the sugar moiety by an esterase, *e.g.* in midecamycin, **11**), or glycosylation at the 2′-OH of the sugar residue.[53, 54]

The gene encoding macrolide 2′-phosphotransferase II was recently found in the plasmid of a clinically isolated *S. aureus* strain.[55] This plasmid also contains two other resistance genes: *msrA*, which encodes protein of an active

efflux pump, and *erm*, which encodes a methyltransferase that modifies an adenine residue of 23S rRNA. Therefore, this strain possesses a collection of all of the three major resistance tools on a single mobile plasmid. A presence of more than one (usually two) resistance machinery is not uncommon, and has been reported in macrolide-resistant enterobacteria[56] and staphylococci.[57, 58]

A self-resistance mechanism involving the modification of macrolides has also been observed in many macrolide-producing organisms. In particular, glycosylation of macrolides to neutralize their biological activity has been detected in 15 out of a total of 32 actinomycete strains producing various polyketide antibiotics.[59] Interestingly, *S. antibioticus,* the oleandomycin producing strain, possesses not only the glycosyltransferase activity that inactivates oleandomycin *via* glycosylation, but also an extracellular β-glucosidase activity capable of removing the added glucose from the modified inactive drug.[60-62] It is thus apparent that *S. antibioticus* utilizes the glycosylation-deglycosylation of oleandomycin as a self-resistance mechanism.[63]

Similar to pathogens, more than one of the above mechanisms is often utilized by macrolide-producing strains for self-protection. This is exemplified by the tylosin producer, *S. fradiae*, employing all of the above strategies: methylation of 23S rRNA,[36, 37] active efflux,[23] and inactivation of tylosin, presumably through its methylation.[64]

1.3 BIOSYNTHESIS OF MACROLIDE ANTIBIOTICS

Macrolide antibiotics consist of a cyclic aglycone of polyketide nature decorated with one or more deoxy sugar moieties. It is generally believed that the

polyketide chain is formed and cyclized first, followed by the attachment of sugar residues, and further modifications of both the aglycone core (*e.g.* hydroxylation) and sugars (*e.g.* methylation). These three stages of macrolide biosynthesis will be discussed in the following sections.

1.3.1 Biosynthesis of Aglycone by Modular Polyketide Synthase

The aglycone of a macrolide is an aliphatic polyketide generated by consecutive condensations of two- or three-carbon units. The resulting β-keto ester after each condensation can be further modified to a β-hydroxy ester, an α,β-unsaturated ester, or a fully reduced unit (Figure 1-3). The macrolactone is biosynthesized by the action of type I polyketide synthases (PKSs), also known as PKS I or modular PKS. PKSs are usually large protein molecules (approximately 300 kDa and higher) consisting of one or more modules, where each module is a functional element responsible for the incorporation and further modification of a single unit of the growing polyketide chain. Each module in turn consists of a few catalytic domains, each responsible for a single chemical transformation. The growing polyketide chain is transferred from one module to another, extended by one polyketide unit with each elongation step, and the whole process is

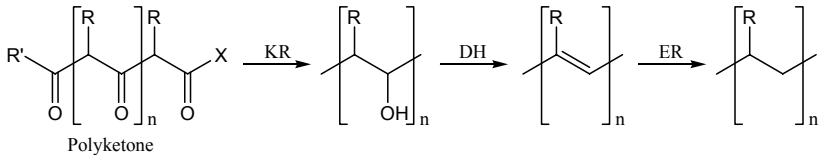

Figure 1-3. General structures of polyketide units: polyketone condensation product and its reduced derivatives. The catalytic domains responsible for the modifications of polyketone, β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER), are discussed in text.

reminiscent of a conveyor assembly. The number of modules determines the size of the macrolide. The heptaketide of erythromycin is biosynthesized by three PKSs: DEBS1 (containing a loading module and modules 1 and 2), DEBS2 (containing modules 3 and 4), and DEBS3 (containing modules 5 and 6), a total of seven modules altogether.

This process of polyketide biosynthesis by modular PKS was first revealed after the discovery of the modular organization of the genes responsible for erythromycin biosynthesis in *S. erythraea*.[65, 66] It is now accepted as a universal strategy for the biosynthesis of all macrolides by modular PKSs. In recent years significant progress has been made in the understanding of the mechanistic details of individual steps.[67-71]

The first step of polyketide synthesis is to prime the loading domain. Each of the PKS modules contains a noncatalytic acyl carrier protein (ACP) domain,

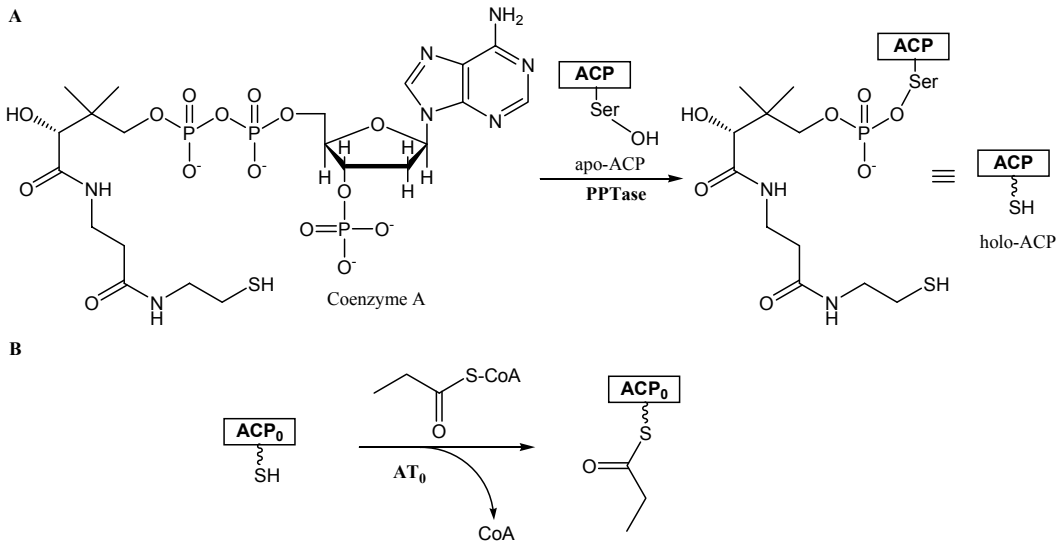

Figure 1-4: Activation of ACP domain by PPTase (A) and chain initiation by AT_0 of the loading domain (B).

which needs to be converted to its active holo form. Specifically, a serine residue of the ACP domain is modified with the phosphopantetheine (Ppant) group derived from coenzyme A (CoA) by the action of phosphopantetheinyl transferase (PPTase) (Figure 1-4 A). The primary thiol group of Ppant will then be acylated with the monomers of the polyketide chain.

The next step is executed by the loading module of PKS consisting of only two domains: acyl transferase (AT) AT_0 and ACP_0. In the case of 6-deoxyerythronolide B, AT_0 catalyzes the acylation of the Ppant thiol group of ACP_0 with a propionyl group using propionyl-CoA (Figure 1-4 B). The action of AT_0 is highly specific to ensure the loading of the appropriate ACP domain with the correct acyl moiety.

The initiation is followed by a series of elongation steps. For each step, the holo-ACP_n domain of module "n" is loaded by AT_n with a methylmalonyl or

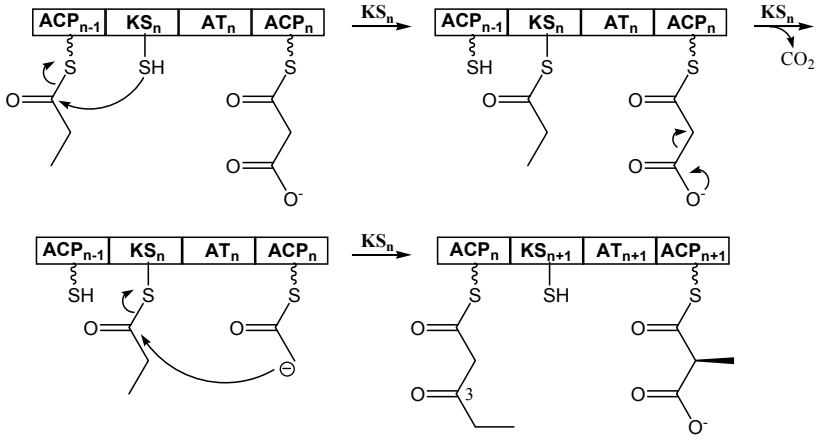

Figure 1-5: Elongation of polyketide chain. Propionyl starting unit is shown attached to ACP_0 (or ACP_{n-1} in general case); malonyl and (2*S*)-methylmalonyl units are shown as acceptors at ACP_n and ACP_{n+1}, respectively.

malonyl moiety derived from methylmalonyl- or malonyl-CoA, respectively. The condensation of the two monomers, one from the donor ACP_{n-1} and another from the acceptor ACP_n, is carried out by the ketosynthase (KS) domain. The acyl moiety of ACP_{n-1} is first transferred to the catalytic cysteine of the KS_n domain. KS_n then decarboxylates methylmalonyl of ACP_n, generating a carbanion, which in turn attacks the acyl moiety on KS_n resulting in the translocation of the extended chain to ACP_n (Figure 1-5). ACP_n now becomes the source of the donor monomer in the next cycle and the process is repeated as many times as needed.

Once the linear polyketide reaches the ACP domain of the last module, the chain termination takes place. The polyketide is transferred to a specific serine of the thioesterase (TE) domain located downstream of ACP, and the thioester is converted into an acylester. A subsequent nucleophilic attack by the proper hydroxyl group (*e.g.* 13-OH in case of 6-deoxyerythronolide B, **15**) of the

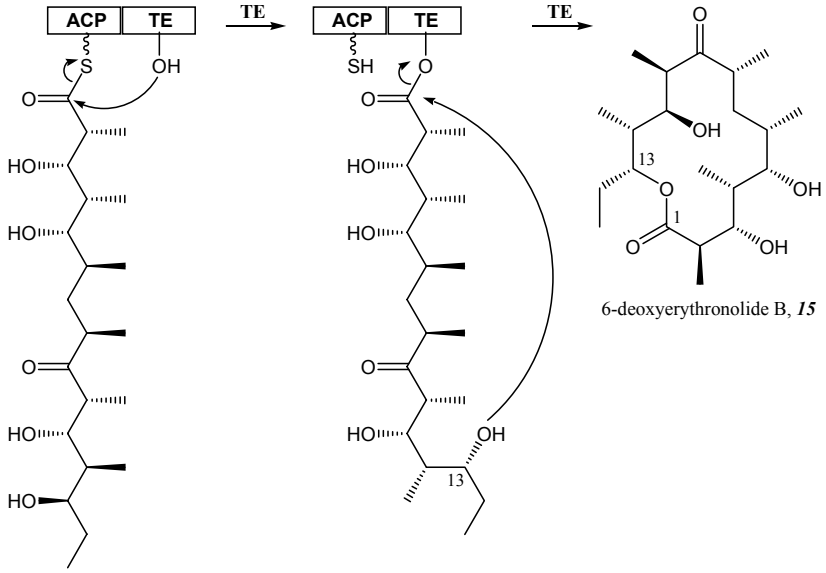

Figure 1-6: Chain termination: example of 6-deoxyerythronolide B biosynthesis.

polyketide chain leads to the cyclization of the linear product, which is then released as the macrolactone (Figure 1-6).

There are many ways in which the great diversity of macrolide structures is achieved by PKS I. A number of acyl starter units can be used for the chain initiation step, including acetyl, propionyl, malonyl, methylmalonyl, and butyryl groups. The units used for the chain elongation are less diverse and are usually malonyl- or methylmalonyl-CoA (2*S*-methylmalonyl-CoA in case of **15**). A final tailor-made polyketide is produced due to the strict specificity of each AT domain for the acyl moiety and the ACP acceptor. The KS domains also show high selectivity for donor and acceptor ACP domains, accounting for the high fidelity of chain transfer between modules. The inter-modular peptide regions (or linkers) within a single PKS and between separate PKS proteins are also known to contribute to the precise biosynthesis of the final product.[72, 73]

Further diversity is achieved when a minimal PKS (KS, AT, ACP) is furnished with one, two or three additional catalytic domains: β-ketoreductase (KR), dehydratase (DH), and enoyl reductase (ER) (Figure 1-3). The KR_n domain stereospecifically reduces the C-3 ketone group of acyl-Ppant-ACP_n to the corresponding β-hydroxy ester using NADPH. It also controls the stereochemistry of the methyl group at the C-2 of the acyl moiety (when methylmalonyl unit is incorporated) by accepting only one epimer as the substrate. In the absence of other domains, the β-hydroxyl group generated by KR will be incorporated into the final polyketide. Alternatively, the hydroxyl group can be eliminated by the action of the DH_n domain, and this dehydration is

induced by the abstraction of the acidic α-proton of the acyl moiety. The resulting $\alpha\beta$-enoyl-Ppant-ACP_n can be further reduced by the ER_n domain *via* a hydride addition using reduced flavin coenzyme, $FADH^-$, to form a fully saturated unit. The presence/absence of these sequentially acting domains in any of the elongation modules will determine the functionalities of the corresponding unit in the final polyketide product.

Perhaps the best studied PKS-catalyzed synthesis is the biosynthesis of erythromycin aglycone, 6-deoxyerythronolide B (**15**), by three DEBS (6-deoxyerythronolide B synthase) proteins (Figure 1-7).[68, 71, 74, 75] (pp. 186-191) Each molecule of **15** is derived from coupling of one propionyl-CoA (starter unit) and six (2*S*)-methylmalonyl-CoA molecules in six elongation cycles. In addition to the minimal PKS domains (KS, AT, ACP), module 4 contains DH, ER, and KR domains that sequentially catalyze the full reduction of the β-ketone to a

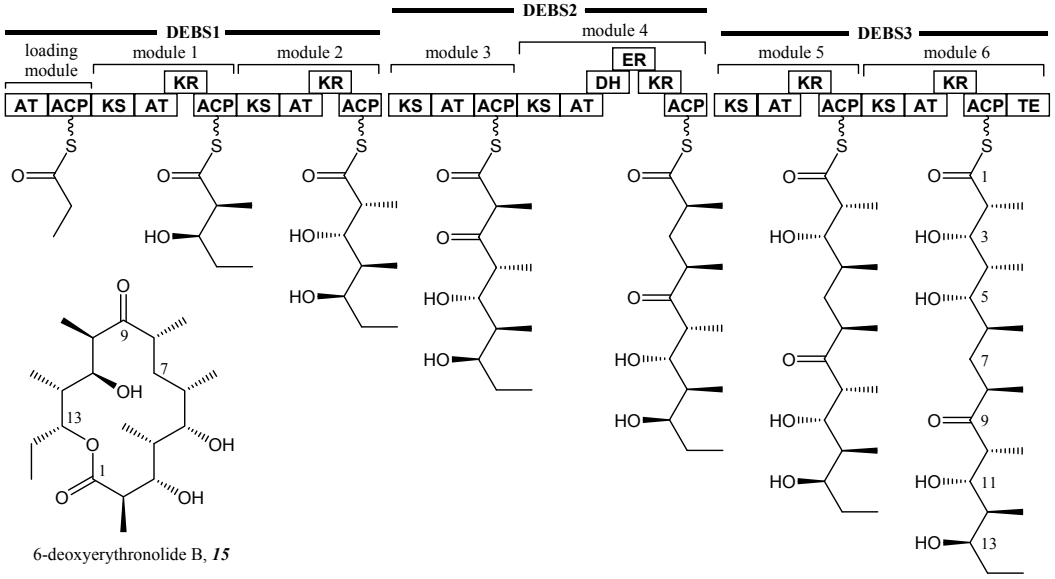

Figure 1-7: The biosynthesis of 6-deoxyerythronolide B by DEBS.

methylene unit (C-7 in the final polyketide). On the other hand, each of the modules 1, 2, 5, and 6, contains an additional KR domain, and therefore the keto group generated in cycles 1, 2, 5, and 6, is only reduced to a hydroxyl functionality (at C-3, 5, 11, and 13), with the specific stereochemistry controlled by KR. Module 3 is a minimal PKS, and consequently the carbonyl group is preserved in the final product (C-9). Six methyl groups at C-2, 4, 6, 8, 10, and 12 are derived from methyl malonyl extension units. The stereochemistry at these positions is thought to be determined by both the presence/absence of an epimerase activity in the corresponding KS domains and the stereoselectivity of the downstream KR domain for the specific epimer at C-2.[68, 76, 77] Finally, TE at the *C*-terminus of module 6 catalyzes the cyclization of the linear polyketide by an attack of the C-13 hydroxyl group at C-1 of the thioester. The release of **15** from PKS frees TE for the next cyclization.

Based on the structural studies of separate DEBS domains, a model for the three-dimensional structure of the DEBS complex has been proposed.[70, 78] According to this model, two identical PKS subunits comprised of linear sequences of domains are associated head-to-head and tail-to-tail and twisted in a helical conformation. The core of the helix is formed by KS, AT and ACP domains, with optional domains (KR, DH, ER) looping out. In such an arrangement, the flexible Ppant linker acts as a "swinging arm", delivering the attached acyl moiety to the corresponding catalytic domains for additional reductive modifications. This model is mainly based on the observation that when isolated individually, KS, AT, and TE domains are homodimers, and

reductive domains (KR, DH, ER) are monomers. It has also been suggested that the two successive PKS homodimers, *e.g.* DEBS1 and DEBS2, interact with each other through complementary 'docking domains' (at the *C*-terminus of DEBS1 and *N*-terminus of DEBS2),[79] facilitating the ordered transfer of the growing polyketide chain between the ACP and KS domains of the two proteins and maintaining the continuity of the DEBS complex.[70, 80]

Alternative models for the three-dimensional structure of DEBS have been proposed, *e.g.* involving head-to-tail homodimerization of DEBS.[81] There is no direct evidence confirming or ruling out any of the models considered, however, the head-to-head model appears to be the most plausible one.[68] To this moment, the only crystal structures available are those of the TE domains of DEBS and pikromycin PKS (PIKS, also referred to as PICS), revealing such common features as "same protein fold, an open substrate channel and a hydrophobic dimer interface".[82, 83]

This example of the biosynthesis of **15** by DEBS illustrates how the size of the polyketide product and its functional groups can be predicted from the domain composition of the PKS (or from the corresponding DNA sequence) responsible for its biosynthesis. However, the polyketide product itself exhibits little or no biological activity and has to be further derivatized, most importantly by glycosylation, in several post-PKS steps. The genes encoding PKS proteins are clustered and are flanked by genes for the biosynthesis and attachment of deoxy sugar(s), genes for other post-PKS modifications, regulatory genes, and resistance determinants.

1.3.2 Biosynthesis and Attachment of Sugar Moiety

The deoxy hexose moieties of macrolide antibiotics are biosynthesized by dedicated genes, often grouped together and usually located in the vicinity of genes coding for PKS. The specific function of each gene is usually assigned based on the similarity of its translated protein sequence to those of known enzymes, and the analysis of the accumulated fermentation products produced by gene knock-out mutants. These methods are useful for establishing the biosynthetic pathway to a specific sugar residue. However, direct enzymatic studies are needed to confirm the function of each enzyme in the pathway and to investigate the mechanistic details of its catalysis. Unfortunately, such thorough analyses are difficult to accomplish in the case of macrolides due to the difficulties in obtaining pure and active enzyme and the often-unstable sugar substrates and pathway intermediates. Recently, a significant amount of information has been accumulated on the biosynthesis of deoxy sugars of lipo-polysaccharides of bacterial cell wall and a few macrolides, *e.g.* erythromycin, pikromycin, and tylosin. The progress has been summarized in a few reviews.[68, 84-90]

The general approach to glycosylation of a macrolide with a deoxy sugar follows the following path: (i) activation of D-glucose-1-phosphate to TDP-α-D-glucose by thymidylyltransferase; (ii) modification of TDP-α-D-glucose by a series of enzymes to the desired deoxy sugar moiety; (iii) coupling of the deoxy sugar to the macrolactone through a nucleophilic displacement of TDP by a

specific glycosyltransferase; and (iv) optional modifications of the sugar moiety, such as methylation by appropriate tailoring enzymes.

Deoxy sugars of macrolides are biosynthesized from D-glucose-1-phosphate (**16**), which is first activated to TDP-α-D-glucose (**17**, sometimes referred to as dTDP-α-D-glucose) by the action of D-glucose 1-phosphate thymidylyltransferase (also known as E_p) (Figure 1-8). TDP-D-glucose is then converted to TDP-4-keto-6-deoxy-D-glucose (**18**) by the NAD^+ dependent 4,6-dehydratase (also known as E_{od}) in a sequence initiated by the oxidation at C-4, followed by the dehydration of C-6, and finally the NADH reduction at C-6. The overall transformation is characteristic of an internal H-transfer: the same hydride that is abstracted from C-4 is returned to C-6 (Figure 1-8). Compound **18** is a common intermediate for the biosynthesis of all deoxy sugars. It is the center of the crossroads where the pathways branch out to form unusual sugars with additional deoxygenations at C-2, 3, or 4, or epimerizations, or amino substitution at various positions. The ketone functionality at C-4 of **18** plays the central role

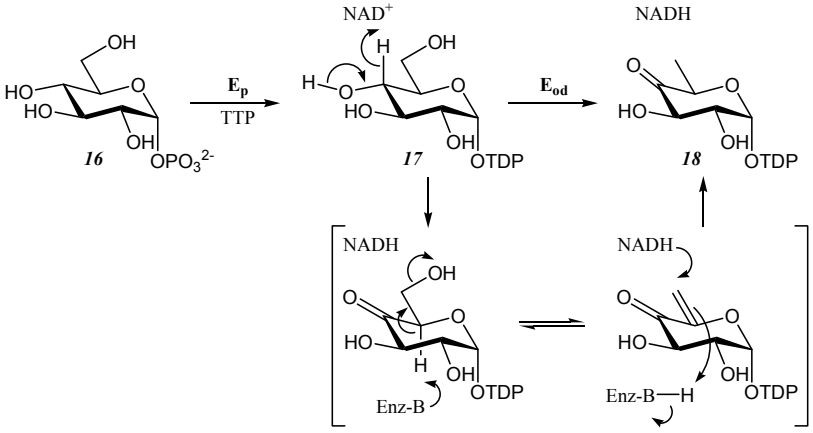

Figure 1-8: Common steps of deoxy sugar biosynthesis catalyzed by D-glucose 1-phosphate thymidylyltransferase E_p and TDP-D-glucose 4,6-dehydratase E_{od}.

in the formation of this diverse array of sugar moieties, and is utilized for the activation of α protons (3-H or 5-H) for the subsequent epimerization or dehydration reactions. It can also facilitate tautomerization or transamination, and finally, after fulfilling its activating function, can undergo a stereospecific reduction (Figure 1-9).

The biosynthesis of desosamine, which is a sugar component of a number of macrolides (Figure 1-1), including erythromycin (**6**) and a set of antibiotics produced by *Streptomyces venezuelae*, **1**-**5**, has been well characterized. The pathway to TDP-D-desosamine (through **23** or **20**, Figure 1-9) was originally proposed based on gene knock-out studies and the sequence homology of the deduced gene products.[91-97] The only step which lacks solid evidence is the conversion of compound **18** to the 3-keto tautomer **23**. This step is speculated to be catalyzed by EryCII in *S. erythraea* (and homologous DesVIII in *S. venezuelae*) mainly based on the fact that the corresponding gene, *eryCII*, is located within the sugar gene cluster and has no close homologues with a confirmed function. The tautomerization of the 4-keto to the 3-keto isomer would allow a deoxygenation at C-4 by a mechanism similar to that for the formation of ascarylose, a 3,6-dideoxy sugar, from **18** by the E_1/E_3 enzyme pair in *Yersinia pseudotuberculosis* (comprehensively reviewed by He and Liu).[88] E_1 is a pyridoxamine 5'-phosphate (PMP)-dependent, iron-sulfur-containing enzyme, while E_3 is a [2Fe-2S]-containing flavoprotein. In the case of desosamine, this transformation would be performed by the action of EryCIV/EryCV or DesI/DesII, the E_1/E_3 homologues in *S. erythraea* and *S. venezuelae*, respectively.

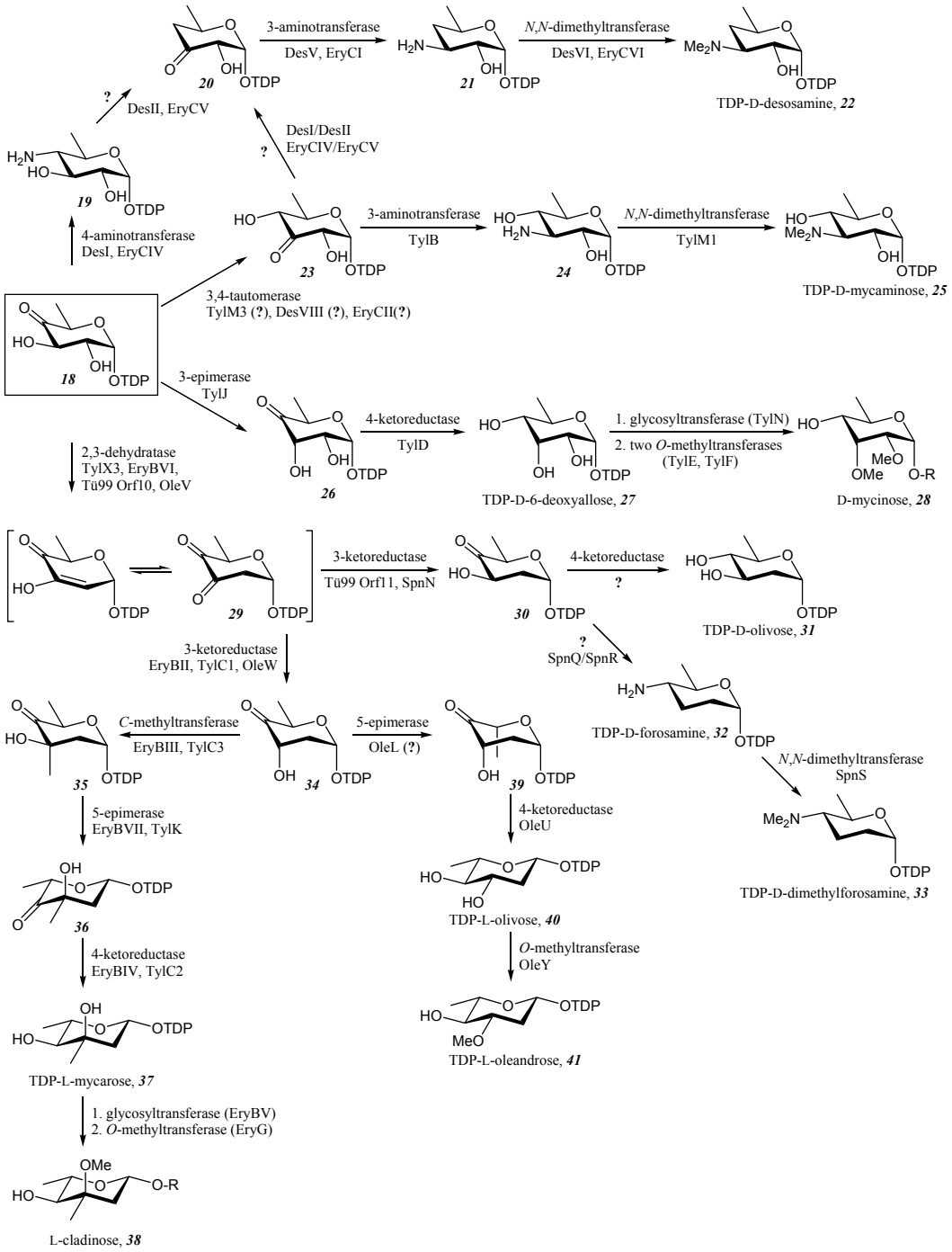

Figure 1-9: Biosynthetic routes to several macrolide deoxy sugars exemplifying the centrality and versatility of common intermediate **18**. The assigned catalytic activities of genes found in the biosynthetic clusters of methymycin (Des) erythromycin (Ery), tylosin (Tyl), oleandomycin (Tü99 and Ole), and spinosin (Spn) are shown. The figure is modified from Ref. 68.

However, based on *in vitro* and *in vivo* experimental results, including the gene knock-out study described in Chapter 2 of this thesis, the route to **20** was revised to include the intermediacy of **19** (Figure 1-9, top portion).[98] The conversion of the intermediate **20** to 3-amino sugar **21** is carried out by a B_6-dependent 3-aminotransferase, EryCI or DesV. The *N*,*N*-dimethylation of **21** by *S*-adenosyl-methionine (SAM)-dependent *N*,*N*-dimethyltransferase DesVI has been demonstrated with the purified enzyme *in vitro*.[99, 100] The final product, TDP-D-desosamine (**22**), is then used as a donor substrate for the dedicated glycosyl-transferase EryCIII or DesVII to make macrolides **1**-**6**.

Unlike **22**, another 3-dimethylamino hexose, TDP-D-mycaminose (**25**), retains the C-4 hydroxyl group, where an additional sugar moiety is attached in several macrolides (Figure 1-2). The tautomer of **18**, compound **23**, is believed to be a pathway intermediate resulting from a tautomerization catalyzed by a homologue of EryCII and DesVIII, TylM3.[101, 102] Compound **23** is then converted to the final product **25** by the action of 3-aminotransferase TylB[103] and *N*,*N*-dimethyltransferase TylM1.[100, 102] The activity of each enzyme has been confirmed by *in vitro* assays. In *S. fradiae*, mycaminose is subsequently attached to the tylosin aglycone, tylactone, by the action of glycosyltransferase TylM2.

The biosynthetic pathway to the second carbohydrate moiety of tylosin, D-mycinose (**28**), has been independently proposed by two research groups and is based on the sequence homology of the corresponding genes to genes with known functions.[104, 105] As shown in Figure 1-9, epimerization at C-3 of **18** by TylJ is followed by a stereospecific reduction of the 4-keto group to a hydroxyl, resulting

in the formation of TDP-D-6-deoxyallose (**27**). After its coupling to mycaminosyltylactone by the glycosyltransferase TylN, the D-6-deoxyallose moiety is methylated at the 2- and 3-OH by the *O*-methyltransferases TylE and TylF, respectively, completing the full conversion to D-mycinose.

The path to the third sugar component of tylosin, L-mycarose, involves the formation of 2-deoxy intermediate **29** which is the product of a 2,3-dehydratase. *In vitro* studies using purified enzymes have shown that the 2,3-dehydratase encoded by *tylX3* of the tylosin producer *S. fradiae*[106] or *orf10* of the oleandomycin producer *S. antibioticus* Tü99[107] catalyzes the formation of **29** in the respective pathways. Compound **29** is then converted in these strains to C-3 epimers **34** and **30** by TylC1 and Orf11, respectively, both of which are NADPH-dependent 3-ketoreductases with opposite stereospecificities. In the case of TDP-L-mycarose (**37**) biosynthesis, **34** is further converted to the branched-chain intermediate **35** by the action of the SAM-dependent *C*-methyltransferase TylC3.[108] The remaining steps are hypothetical and presumably consist of C-5 epimerization by TylK, followed by TylC2 catalyzed 4-ketoreduction to form **37**. The attachment of mycarose to the diglycosylated tylosin precursor by the glycosyltransferase TylCV completes the biosynthesis of tylosin.

A methylated derivative of mycarose, L-cladinose (**38**), is found in the structure of erythromycin. A set of genes homologous to those dedicated to the mycarose biosynthesis in the tylosin cluster can also be found in the erythromycin gene cluster.[92-94] Although no biochemical studies of the respective enzymes have been done, it is likely that the pathway to mycarose is the same as in the

tylosin case. The attachment of L-mycarose to the erythromycin aglycone **15** is believed to be catalyzed by glycosyltransferase EryBV. This is followed by attachment of the second sugar, desosamine, and the hydroxylation of the aglycone portion at C-12.[68] The methylation of the 3-OH of L-mycarose by EryG takes place after all of these steps and completes the biosynthesis of the L-cladinose moiety.[109]

The mycarose precursor **34** is also an intermediate in the pathway to TDP-L-oleandrose (**41**), and its precursor, TDP-L-olivose (**40**).[69, 107, 110, 111] It has been demonstrated that glycosyltransferase OleG2 from the oleandomycin (**9**) pathway is capable of coupling both **40** and **41** onto the erythromycin aglycone **15**.[110] However, the substrate for the *O*-methyltransferase OleY is L-olivosyl-erythronolide B rather than the free sugar **40**.[112] Therefore, it is likely that the glycosylation precedes the *O*-methylation. Interestingly, in the biosynthesis of the macrolide avermectin by *Streptomyces avermitilis*, **41** is formed from **40** first and is then transferred to the aglycone.[113, 114]

The C-3 epimer of **34**, compound **30**, has been proposed to be an intermediate in the biosynthesis of TDP-D-dimethylforosamine (**33**) which is a part of the polyketide insecticide spinosyn. The key step is the conversion of **30** to the 2,3,4,6-tetradeoxy-4-amino sugar **32** catalyzed by SpnQ/SpnR.[115] The mechanism of this intriguing transformation remains a mystery. The D-dimethylforosamine is also a structural component of spiramycins (*e.g.* **12**, Figure 1-2). Meanwhile, C-4 reduction of **30** would yield TDP-D-olivose (**31**), which is found as a part of aromatic polyketides including mithramycin, urdamycin, and

landomycin, and is also found in macrolides, such as chlorothricin (*S. antibioticus*).

1.3.3 Other Tailoring Steps

The most common post-PKS modifications, other than glycosylations, are catalyzed by oxidoreductases and methyltransferases. The former introduce hydroxyl, aldehyde, or epoxide functionalities in the aglycone portion, and the latter modify side chains of both the aglycone and the sugar components. These modifications often change the solubility of the molecule, alter the pattern of hydrogen donor/acceptor sites, impose steric restrictions, and introduce reactive functional groups (*e.g.* aldehyde) or 'handles' for further modifications (*e.g.* hydroxyl).[116] They can take place before or after glycosylation steps. The genes encoding enzymes for post-PKS tailoring are usually clustered with PKS and sugar biosynthetic genes.

The oxidoreductases involved are usually highly specific heme-containing cytochrome P450 monooxygenases. It is well known that P450 enzymes require additional reducing enzymes for ferrous iron regeneration. However, the genes encoding these P450 reductases are usually located outside the macrolide biosynthetic gene cluster. They can be replaced by components from other P450 systems in *in vitro* assays.

In the biosynthesis of erythromycin A the first protein-free PKS product **15** is hydroxylated at C-6 by hydroxylase EryF to form erythronolide B (**42**), which is glycosylated with the first sugar component, mycarose, by the action of EryBV (Figure 1-10). Subsequent catalysis by glycosyltransferase EryCIII leads

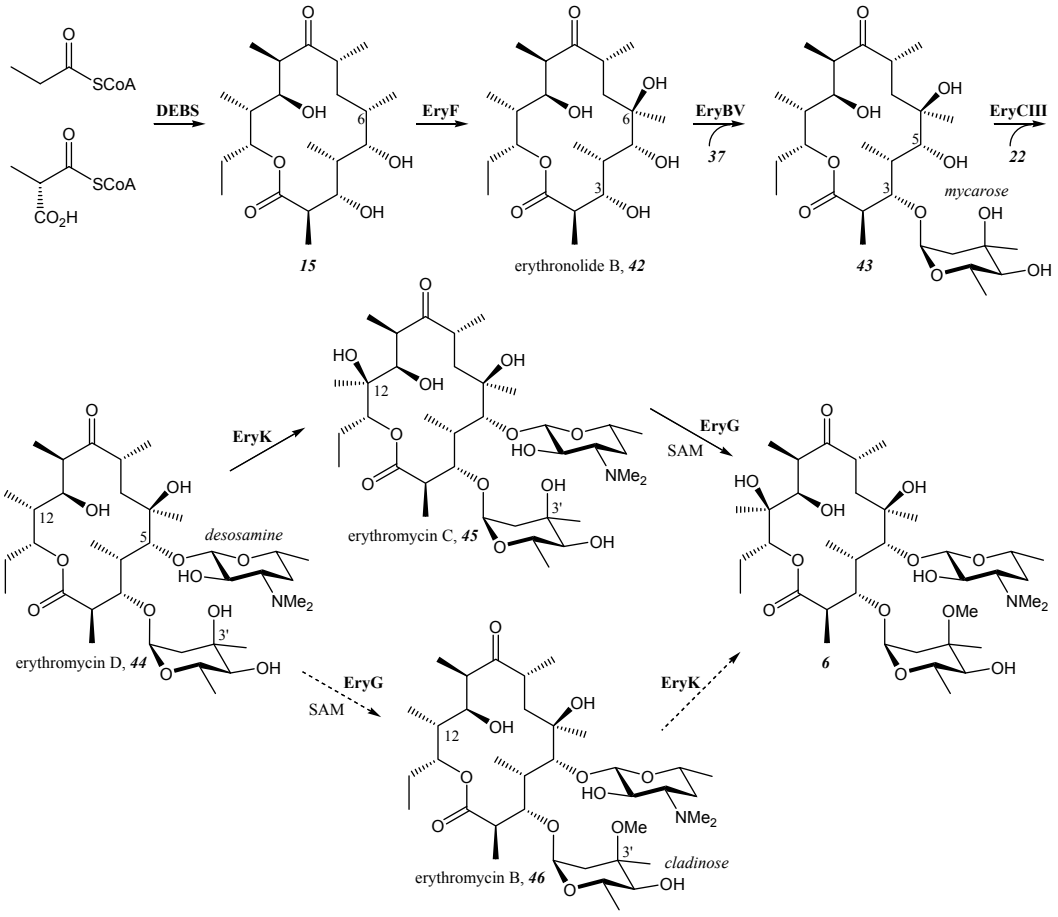

Figure 1-10: Post-PKS steps in the biosynthesis of erythromycin A (**6**).

to the addition of the desosamine residue at C-5 of the monoglycosylated **43**, generating erythromycin D (**44**). The second hydroxylase, EryK, introduces a hydroxyl group at C-12 of **44** to give erythromycin C (**45**). The mycarose residue of **45** is then converted to cladinose by the action of the SAM-dependent *O*-methyltransferase EryG, completing the biosynthesis of **6**. A mutation of *eryK* in *S. erythraea* led to the accumulation of both erythromycin B (**46**) and erythromycin D (**44**), indicating a possible reverse of the order of the methylation

and hydroxylation steps.[117] It was later determined that EryG could act upon both **44** and **45**, with a 2-fold preference toward **45** over **44**.[109] The final conclusion, however, was made on the basis of *in vitro* studies in which EryK was shown to have a strong preference for **44** over **46** (greater than 1200-fold).[118] Thus, methylation by EryG is clearly the last step in the biosynthesis of **6,** and **46** is merely a shunt product.

The sequence of the post-PKS steps in the biosynthesis of tylosin (**13**, see Figure 1-11 for the preferred pathway) is more ambiguous.[116, 119] It begins with the TylM2 catalyzed addition of mycaminose to the tylactone (**47**) followed by oxidation at C-20 and C-23 by dedicated P450 monooxygenases TylI and TylH1, respectively. The hydroxylation at C-23 provides a site in **49** for the second glycosylation with 6-deoxyallose by the glycosyltransferase TylN. This is followed by the attachment of the last sugar residue, mycarose, by TylC5. This described order of glycosylation has not been fully established, and the last two steps may be reversed. Mycarose remains intact in the final product, whereas 6-deoxyallose is further modified by two *O*-methyltransferases, TylE and TylF, completing the construction of the final product **13**.[104, 120, 121]

The sequence of the post-PKS steps of the oleandomycin (**9**) biosynthesis was recently established by expressing the P450 monooxygenase OleP and two glycosyltransferases, OleG1 and OleG2, isolated from the oleandomycin producer *S. antibioticus* in a *S. erythraea* mutant.[122, 123] It was confirmed that OleP is indeed responsible for the formation of the epoxide moiety of **9**. It was also found that the epoxide formation and the coupling of the first sugar moiety, olivose, by

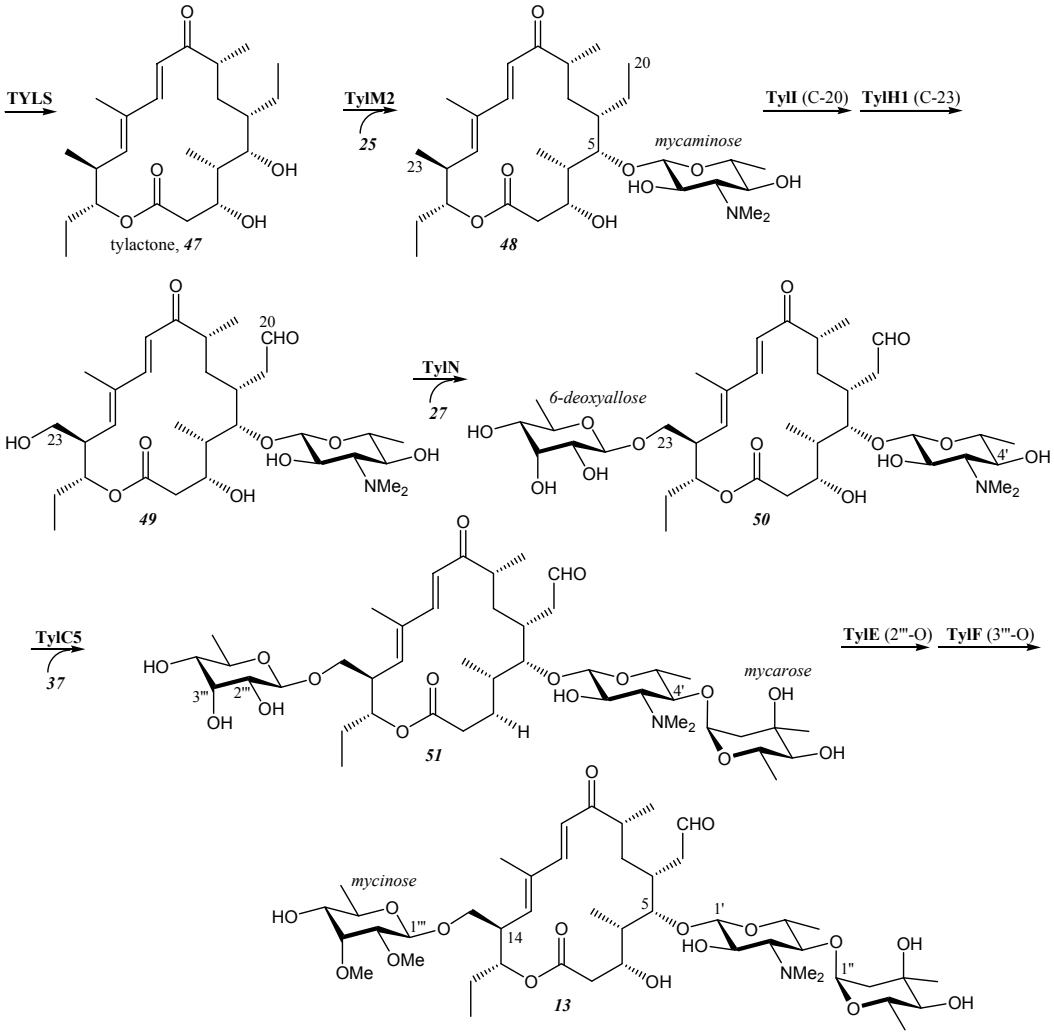

Figure 1-11: Post-PKS steps in the biosynthesis of tylosin (**13**).

OleG2 can occur in random order.[123] The final step involves methylation of the olivose moiety attached to the aglycone into oleandrose by the methyltransferase OleY, followed by the second glycosyl transfer catalyzed by OleG1 to form **9**.[112]

The *O*-methylation of the aglycone portion of polyketides is more common for aromatic polyketides, but also occurs in macrolides. A good

example is the macrolide antibiotic FK-506 produced by *Streptomyces* sp. MA6858, in which the methylation is catalyzed by a SAM-dependent methyl-transferase.[124] An alternative way to incorporate an *O*-methyl group in the macrolides is by using the methoxymalonate unit during the polyketide synthesis as exemplified by the biosynthesis of **11**, **12**, and **14**.[69]

Another type of post-PKS modification is acylation of hydroxyl groups of the aglycone or the sugar moiety by acyltransferases. For example, MdmB catalyzes the acetylation and the propionylation of the 3-OH of mideacamycins (*e.g.* **11**) produced by *Streptomyces mycarofaciens*.[125] Likewise, acyltransferase CarE catalyzes the conversion of mycarose to isobutyrylmycarose found in carbomycins (*e.g.* **14**).[126]

1.4 ENGINEERING NOVEL MACROLIDES

It has become clear in recent decades that the biosynthetic machineries of macrolides can be manipulated to create analogs with diverse polyketide as well as sugar structures. These types of studies are fueled by the urgent need for new drugs for treatment of bacterial infections caused by pathogens resistant to known antibiotics. This topic has been extensively reviewed in recent literature and will only be discussed here in brief.[127-130]

The modular nature of PKS and the colinearity between the PKS domains and the structural components of polyketide products provide an excellent basis for creating structural diversity by gene manipulation. This approach relies on the promiscuity of some PKS domains and is complemented by the exogenous

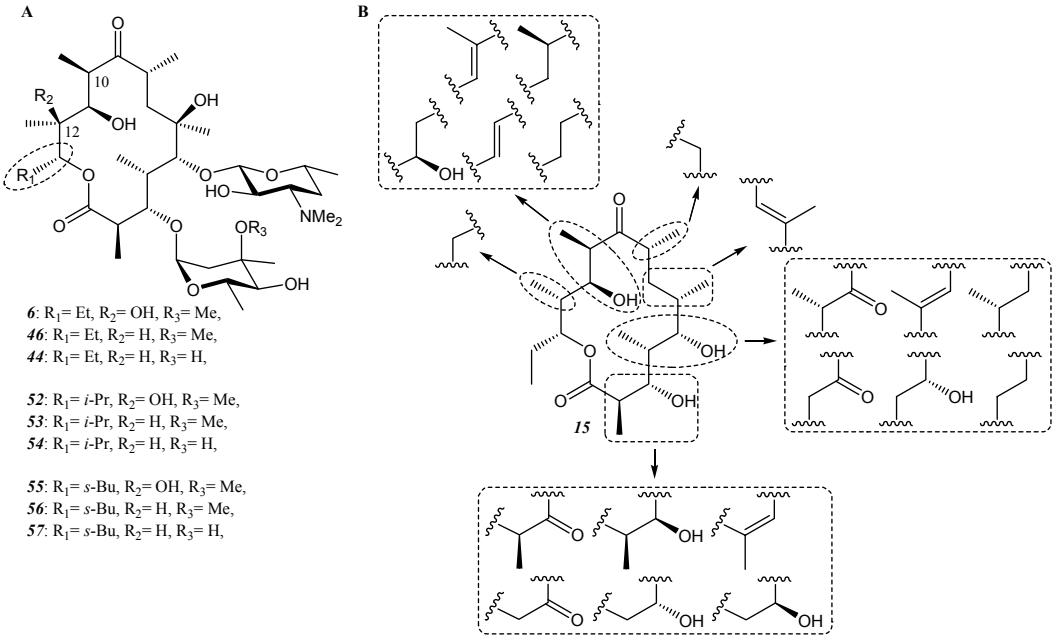

Figure 1-12: Examples of modifications of erythromycin aglycone through A: incorporation of alternative starter units, and B: alterations of chain extension steps.

addition of "unnatural" (that is, not normally used by specific enzyme) building blocks or intermediates.

For example, the incorporation of acyl moieties other than propionyl into erythromycin analogs during the chain initiation step has been demonstrated when the loading module of DEBS1 in *S. erythraea* is replaced by the more promiscuous loading module of the avermectin PKS. Six new analogs of erythromycins A, B, and D (**6**, **46**, and **44**), compounds **52**–**57**, were produced by this mutant (Figure 1-12A).[131] The diversity of such substitutions, however, is limited by the availability of the acyl-CoA substrates in the cell.

As mentioned earlier, the incorporation of the specific extension unit, malonate or methylmalonate, is determined by the corresponding AT domain.

Therefore, the addition or removal of a specific methyl group can be achieved by the replacement of the malonate-specific AT with the methylmalonate-specific version or vice versa. For example, the replacement of methylmalonate-specific ATs in modules 1 and 2 of DEBS1 with known malonate-specific domains resulted in the accumulation of erythromycin analogs lacking methyl substituents at C-12 and C-10, respectively.[132]

Inactivation of specific KR, DH, and ER domains or their replacement with counterparts from other modules of the same PKS (or PKS of different pathways) allows manipulation of the stereochemistry (*R*- or *S*-hydroxyl, *E*- or *Z*-double bond) and the oxidation state (ketone, alcohol, double bond, or methylene) at a specific β-carbon. A library of over 50 analogs of the erythromycin aglycone **15** was generated by substituting AT, KR, DH, and ER domains of DEBS with those of the rapamycin PKS.[133] Functional groups at up to three carbon centers in a single compound could be changed simultaneously. Figure 1-12B illustrates the substitutions that were introduced.

The size of the macrolide ring can also be altered by the repositioning of the TE domain. When the TE domain of DEBS was moved to the end of DEBS1, a shorter polyketide chain was released, and therefore, lactones with a smaller ring size were obtained.[134] A recently discovered natural phenomenon named 'stuttering' could be of advantage for creating macrolides with a bigger ring size. Wilkinson *et al.* have found that module 4 of DEBS occasionally operates twice resulting in a 16- instead of a 14-membered ring final product.[135]

Owing to the importance of the carbohydrate residues for the biological activities of macrolides, the manipulation of sugar structures has drawn much attention in recent years.[86, 116, 136, 137] Modified sugar residues are constructed separately by the sugar biosynthetic machinery and then incorporated as a part of the macrolide structure. This approach relies on the relaxed substrate specificity of the corresponding glycosyltransferase. A glycosyltransferase suitable for the combinatorial biosynthesis should be able to couple "unnatural" aglycones with "unnatural" carbohydrates.

The simplest way to alter the glycosylation pattern of a macrolide is the inactivation of the specific glycosyltransferase gene, thereby preventing the attachment of the corresponding sugar moiety. For example, the inactivation of each of the glycosyltransferase genes of the tylosin biosynthesis led to the accumulation of tylosin derivatives with different glycosylation patterns (Figure 1-11).[119, 138, 139] This tactic is usually employed for the elucidation of the order of glycosylation steps and the function of the particular glycosyltransferase during the biosynthesis of the macrolides containing two or more carbohydrate residues.

Generation of an array of "unnatural" structures requires manipulation of the sugar biosynthetic machinery directly. The best-studied case is the alteration of the biosynthetic pathway for desosamine in the methymycin/pikromycin set of antibiotics, and it will be discussed in Section 1.5 and Chapter 2. Alternatively, inactivated genes can be replaced with genes from different pathways and/or species (Chapter 2). The development of suitable genetic tools has greatly facilitated the construction of hybrid natural products. For example, shuttle

vectors are now available that can be transferred from *E. coli* to *Streptomyces* species, and are capable of replicating in both species.[140] Sugar biosynthetic genes have been cloned in such vectors under the control of strong *Streptomyces* promoters and are either integrated into the chromosome of the new host or are expressed from the plasmid.

In the study by Cundliffe and coworkers, two genes of the desosamine pathway in the narbomycin producer *Streptomyces narbonensis*, *nbmK* and *nbmJ* (homologues of *eryCIV* and *eryCV*, respectively, see Figure 1-9), were introduced into the tylosin producer *S. fradiae*.[141] This transformation led to the production of TDP-D-desosamine in addition to TDP-D-mycaminose. Consequently, two new compounds, **58** and **59**, were detected, both containing desosamine (Figure 1-13A). The incorporation of desosamine in the final products indicates that the glycosyltransferase TylM2 in the tylosin pathway is capable of processing desosamine as well as its natural substrate, mycaminose.

In the study described above, researchers took advantage of the natural production of the aglycone by *S. fradiae*. Alternatively, McDaniel and Tang used *S. lividans*, which cannot make macrolactones by itself, as their host strain.[142] They integrated all of the genes necessary for the biosynthesis and transfer of desosamine into the chromosome of *S. lividans*. The resulting strain was then transformed with the library of plasmids expressing modified DEBS genes and directing the production of different 14-membered macrolides (derivatives of **15** shown in Figure 1-12B). Although the yields of glycosylated products were low, more then 20 desosaminylated macrolides with detectable activity against

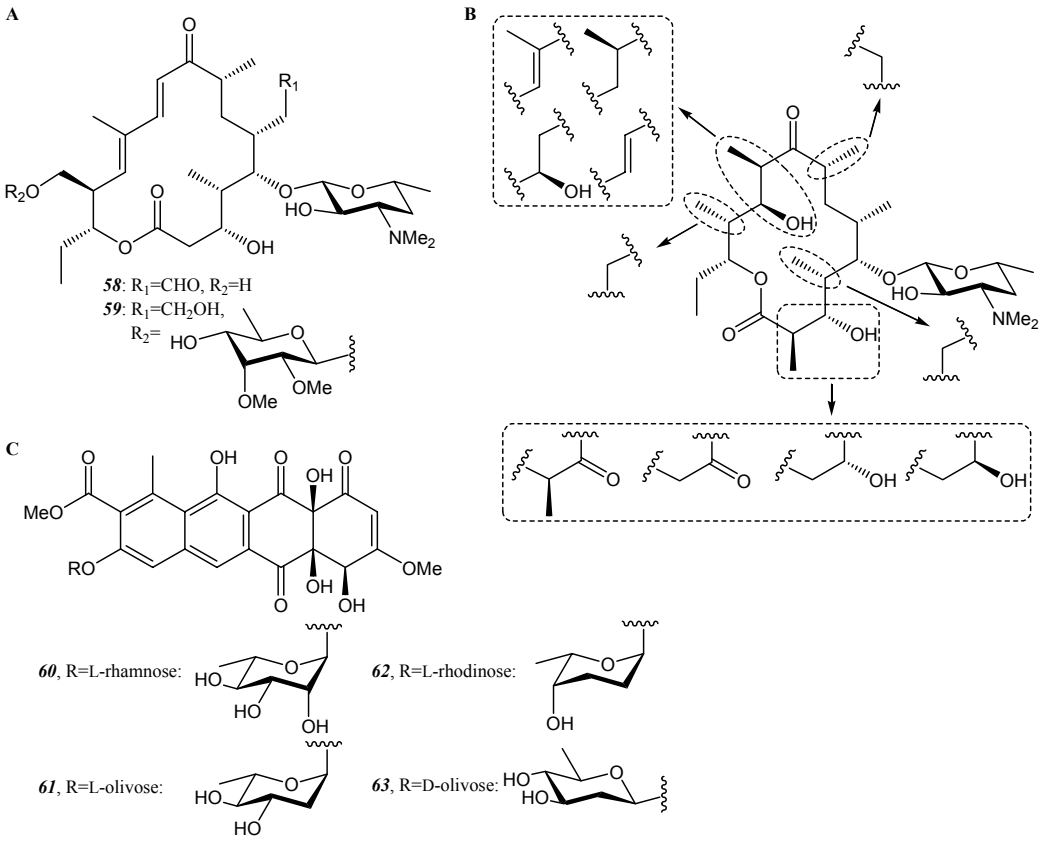

Figure 1-13: Examples of glycosylated compounds generated by manipulations of sugar biosynthetic genes.

Bacillus subtilis were identified by mass spectroscopy (Figure 1-13B).[142] This case nicely demonstrates the flexibility of the responsible glycosyltransferase, DesVII, towards its aglycone substrates.

Likewise, Salas and coworkers have also employed the 'sugar flexible' glycosyltransferase of the aromatic polyketide elloramycin biosynthesis, ElmGT, for the glycosylation of an aromatic aglycone with diverse deoxysugars.[143] A plasmid pLN2 containing the genes required for the formation of TDP-L-oleandrose from the oleandomycin (**9**) pathway was created first. This plasmid

was further modified by the gene deletion and replacement to create sets of genes for the biosynthesis of TDP-L- and -D-olivose, TDP-L-rhamnose, and TDP-L-rhodinose. Derivatives of plasmid pLN2 were then used to complement a *Streptomyces albus* mutant expressing the *elmGT* gene. The aglycone was either supplemented by feeding or produced endogenously from the cosmid containing tetracenomycin C biosynthetic genes. The detection of four glycosylated derivatives of tetracenomycin C, **60**–**63** (Figure 1-13C), confirmed the production of the desired sugar residues and illustrated the versatility of the glycosyl-transferase ElmGT.[143] The plasmids created in this study can potentially be used in other *Streptomyces* strains for the attachment of sugar residues to different aglycones provided these hosts contain a flexible glycosyltransferase.

However, the above *in vivo* engineering of novel glycosylated products may be complicated by cell death or cell growth inhibition since the new macrolides may be toxic. A chemoenzymatic approach to the production of glycosylated secondary metabolites by carrying out the glycosyl transfer step *in vitro* has been developed by Thorson and coworkers.[144] The strategy, termed glycorandomization, takes advantage of the versatility of the chemical synthesis to make diverse sugar structures and the promiscuity of sugar kinases and nucleotidylyltransferases to create activated carbohydrate building blocks.[145] Coupled with a substrate flexible glycosyltransferase, glycorandomization potentially allows the construction of a large library of glycosylated molecules *in vitro* (Figure 1-14). Thorson and coworkers have demonstrated the viability of this approach by creating 21 monoglycosylated vancomycin analogs using the

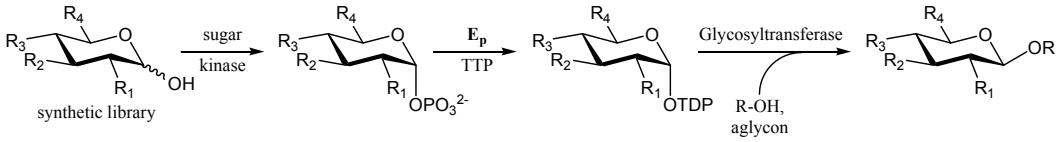

Figure 1-14: Schematic illustration of glycorandomization approach to new glycosylated products.

vancomycin aglycone, a library of sugar-1-phosphates, and the glycosyl-transferase of the vancomycin biosynthesis, GtfE.[146] The attempt to glycosylate the aminocoumarin aglycone using the same library of carbohydrate substituents and the glycosyltransferase of the novobiocin biosynthesis, NovM, was less successful resulting in only four glycosylated products.[147] Perhaps the varied result reflects the degree of promiscuity exhibited by the respective glycosylating enzymes. Future protein engineering to make a fully flexible glycosyltransferase or a glycosyltransferase with the desired specificity would allow further realization of the potential of this combinatorial approach. Alterations of post-PKS steps other than the glycosyl transfer reaction are not as common.[116]

In summary, advances have been made in engineering new macrolides with different aglycone structures and glycosylation patterns. Gene manipulations *in vivo* have proven to be an effective means to create structural diversity. The coupling of the aglycone and the sugar *in vitro* allows the incorporation of a great variety of sugar structures to achieve glycodiversification. It also avoids the potential toxicity of the new "drug" to the producing host. It is likely that a combination of these methods, *e.g.* generating a library of aglycones through genetic variations and coupling it with a library of synthetic sugar nucleotides *in vitro*, is the most efficient route to novel structures.

1.5 BIOSYNTHESIS OF MACROLIDES BY *S. VENEZUELAE*

S. venezuelae ATCC 15439 produces a set of structurally and biosynthetically related 12- and 14-membered macrolide antibiotics, each containing a single deoxysugar moiety, D-desosamine. The prevalence of a group of macrolides having the same ring size is growth media dependent: the 14-membered pikromycin (**4**) and narbomycin (**5**) are isolated from the cultures grown in PGM medium,[148] whereas the 12-membered methymycin (**1**), neomethymycin (**2**), and their non-hydroxylated precursor, YC-17 (**3**) are produced by cultures grown in SCM[149] or seed/vegetative[150] media (Figure 1-1). The reasons why two sets of related macrolides with different ring size are produced by *S. venezuelae* has been puzzling since these compounds were first identified.

Our group, in collaboration with Professor Sherman, has sequenced the entire gene cluster for the biosynthesis of methymycin/pikromycin in *S. venezuelae*.[95] The initial assignment of the gene functions is based on the sequence comparison with genes found in the erythromycin and oleandomycin biosynthetic gene clusters.[151, 152] The sequence analysis has revealed the presence of five open reading frames (ORFs) coding for the modular PKS proteins, *pikAI*, *pikAII*, *pikAIII*, *pikAIV*, and *pikAV* (Figure 1-15). These are followed by the cluster of genes involved in deoxysugar biosynthesis and attachment, *des*. The ORF encoding a cytochrome P450 monooxygenase, *pikC*, is located downstream of the *des* genes, and is followed by a putative regulatory gene, *pikD*. Upstream of *pikAI* there exist two MLS_B type resistant genes, *pikR1* and *pikR2*, which likely

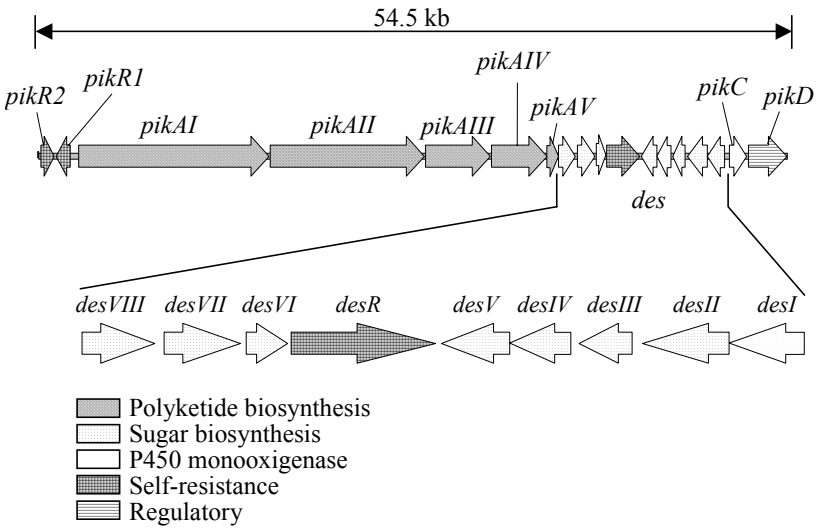

Figure 1-15: Organization of methymycin/pikromycin biosynthetic gene cluster in *S. venezuelae*.

play roles in the self-resistance mechanism *via* rRNA methylation. Interestingly, within the *des* cluster there also exists an ORF, *desR*, encoding a protein homologous to β-glycosidase. This β-glycosidase was speculated to be involved in an alternative self-resistance mechanism through the glycosylation-deglycosylation of the antibiotic.[62]

Since the presence of *desR* in the desosamine biosynthetic cluster is intriguing, its function was further investigated by gene disruption experiments. Our results showed that the inactivation of *desR* in *S. venezuelae* genome resulted in the accumulation of two new glycosylated products in addition to **1** and **2**.[153] These products were identified as derivatives of **1** and **2** with an additional glucose moiety attached at the 2′-OH of the desosamine moiety. As expected, these new products have no antibiotic activity. The study thereby provided strong

evidence supporting the function of DesR as a β-glycosidase, which is likely involved in the glycosylation-deglycosylation self-resistance mechanism in *S. venezuelae*. However, this appears to be a secondary mechanism, since **1** and **2** remained to be the major products produced by the *desR* mutant. Hence, methylation of rRNA by PikR1 and PikR2 may still be the primary self-resistance strategy.

The organization and functions of the PikA domains are depicted in Figure 1-16. The loading module of PikAI contains a KS-like domain, KS^Q, not present in DEBS, but typical for other modular PKSs. It has been shown that KS^Q possesses a decarboxylase activity[154] and is likely involved in the tandem formation of the propionyl starter unit from methylmalonyl-CoA. The AT domain of module 2 is malonate-specific, whereas the remaining AT domains, including that of the loading module, are methylmalonate-specific. The KR

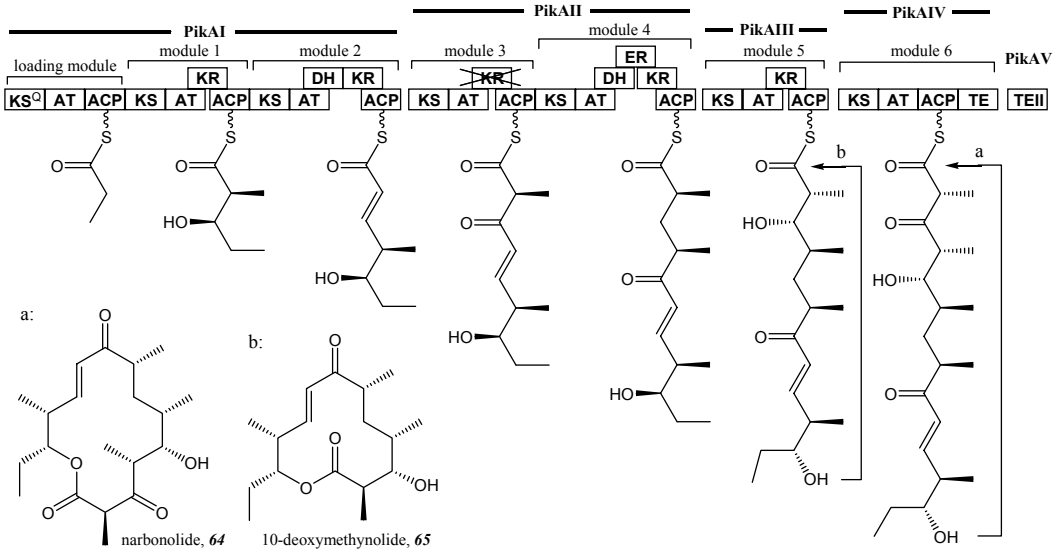

Figure 1-16: Polyketide biosynthesis by PIKS of *S. venezuelae*.

domain of module 3 is inactive, resulting in the retention of the ketone moiety, C-9 of **64** and C-7 of **65**, in the final product.

An interesting property of pikromycin PKS (PIKS) is that its modules 5 and 6 are separate proteins, unlike those of DEBS and oleandomycin PKS, where they are parts of a single bimodular protein. This peculiarity has been associated with the production of macrolides of two ring sizes, **64** and **65**, by a single PIKS.[95] Furthermore, in addition to a 'conventional' TE domain at the *C*-terminus of PikAIV, PIKS has a second TE, TEII, encoded by a separate ORF *pikAV*. Therefore, it has been initially suggested that a longer polyketide results from the operation of the entire PIKS, culminating in the chain termination by TE and the release of **64**. Alternatively, the 12-membered cyclic product **65** is formed when only modules 1 through 5 are used, and the polyketide is prematurely released by TEII (Figure 1-16).[95] However, a *S. venezuelae* mutant lacking the *pikAV* gene produced decreased amounts (<5%) of both 12- and 14-membered macrolides,[95] suggesting that TEII is not the key to the alternative chain termination and it plays a more general role in macrolide biosynthesis. This general hypothesis is supported by the existence of *pikAV* homologues in many polyketide and nonribosomal peptide biosynthetic gene clusters where a single product is produced by the respective biosynthetic machinery.[155] It has been hypothesized that TEII may have an editing function, which is to remove aberrant intermediates from the polyketide assembly line, thereby preventing it from stalling.[156] However, the exact role of the thioesterase TEII remains to be elucidated.

Later study by Xue and Sherman confirmed that the release of macrolactones of both ring sizes is executed by the TE domain of PikAIV.[157] It was found that, depending on the culture conditions, two different forms of PikAIV can be produced by *S. venezuelae*. Under the conditions for the production of 14-membered **64**, the full length PikAIV is produced, and PIKS functions in the usual manner. In contrast, under the conditions for the production of 12-membered **65**, the *pikAIV* gene is translated from the alternative start codon to express the *N*-terminally truncated PikAIV, lacking roughly half of its KS_6 domain. Xue and Sherman proposed a structural model where the TE domain of the truncated PikAIV occupies the space usually taken by KS_6. This allows a direct transfer of the hexaketide from ACP_5 to TE, bypassing module 6 and resulting in the cyclization of a shorter polyketide chain.[157] Further investigation by Sherman and coworkers led to the proposal of another mechanism of the chain transfer where the hexaketide is first transferred from ACP_5 to ACP_6 of the truncated PikAIV, followed by the relocation to the active serine of TE and the release of a 12-membered cyclic product.[158] This last model represents our current understanding of the dual function of PIKS in the production of two related but different macrolides.

Meanwhile, sequence analysis of the *des* gene cluster in conjunction with the comparison to the desosamine biosynthetic genes found in the erythromycin producer *S. erythraea*,[92, 93] allowed a tentative function assignment of the desosamine biosynthetic genes in *S. venezuelae* (Figure 1-17).[95] An early study showed that the deletion of both *desV* and *desVI* genes abolished the production

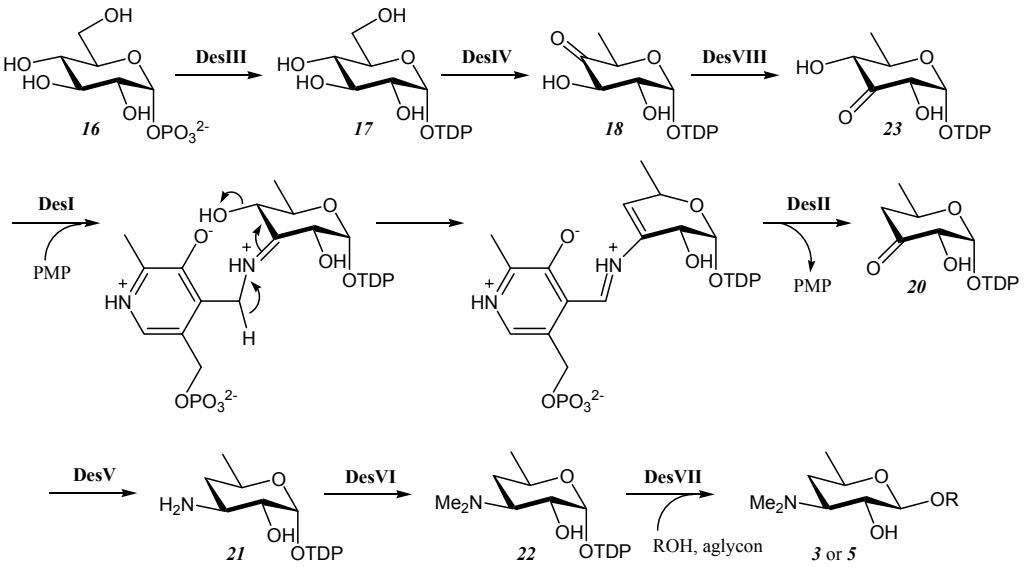

Figure 1-17: Proposed scheme for the biosynthesis and attachment of TDP-D-desosamine (**22**) in *S. venezuelae*.

of **1**, **2**, **4**, and **5**. The major products produced by these two mutants are the aglycones **64** and **65**. These results indicate that there exists only one set of genes for the biosynthesis of desosamine which is used to glycosylate both 12- and 14-membered macrolides.[95]

Further studies showed that the first two steps of desosamine biosynthesis are the same as for the biosynthesis of other deoxysugars and are catalyzed by DesIII and DesIV (Section 1.3.2).[84] DesVII shows a significant sequence similarity to several previously identified glycosyltransferases, *e.g.* TylM2 from the tylosin cluster in *S. fradiae*,[101] EryCIII and EryBV from the erythromycin cluster.[92, 93] These enzymes catalyze the coupling of deoxysugars and polyketide aglycones in their respective pathways. Therefore, DesVII was assigned as a desosaminyltransferase that catalyzes the addition of desosamine onto **64** and **65**.

This DesVII-catalyzed glycosyl transfer should be one of the last steps in the pathway.

The nature and the order of the transformations between these steps are less obvious. The mechanism of the C-4 deoxygenation step is especially intriguing, which has been proposed to be catalyzed by a pair of enzymes, DesI and DesII. DesI shows a significant sequence similarity to several known enzymes, including the well-characterized E_1 enzyme from the CDP-L-ascarylose biosynthetic pathway in *Yersinia pseudotuberculosis*.[84] E_1 is a PMP-dependent enzyme. When coupled with the reductase E_3, E_1 catalyzes the C-3 deoxygenation of **18** during the formation of 3,6-dideoxyhexoses.[159-162] However, no E_3 homologue exists in the *des* cluster. Only the *desII* gene, whose translated sequence shows characteristics of a [4Fe-4S] protein, likely encodes a reductase, performing the reaction analogous to that catalyzed by E_3. Therefore, the C-4 deoxygenation in desosamine has been proposed to be catalyzed by DesI and DesII following a similar mechanism as C-3 deoxygenation catalyzed by E_1 and E_3.[97] If the proposed mechanism of C-O bond cleavage at C-4 is correct, an isomerization of the 4-keto-6-deoxyhexose **18** to its 3-keto derivative **23** becomes a prerequisite. Such a tautomerization is speculated to be catalyzed by DesVIII which shows modest sequence homology to P450 enzymes but lacks the conserved residues for heme binding (Figure 1-17).[153]

The next two steps, the introduction of an amino group at the C-3 position and the subsequent *N*,*N*-dimethylation, are catalyzed by DesV and DesVI, respectively. The functions of the *desV* and *desVI* genes have been confirmed by

gene disruption experiments[96, 97] as well as *in vitro* biochemical studies.[99, 100, 163] In these studies, two mutants of *S. venezuelae*, KdesV and KdesVI, were constructed that had *desV* and *desVI* genes disrupted, respectively.[96, 97] When they were fermented under the 12-membered ring production conditions, in both cases, no metabolites containing desosamine were detected. Instead, small quantities of new glycosylated products were obtained.

A fermentation of KdesV led to the isolation of two methymycin/ neomethymycin analogs, **66** and **67**, each carrying a 4,6-dideoxy hexose (Figure 1-18). These results strongly supported the assignment of *desV* as the gene encoding the 3-aminotransferase that catalyzes the conversion of the 3-keto-sugar **20** to the corresponding amino sugar **21**. The deletion of this gene prevents the C-3 transamination, resulting in the accumulation of **20**. The production of **66** and **67** (which have a hydroxyl group at C-3 of the sugar moiety instead of the expected keto group) indicates the existence of a pathway-independent reductase

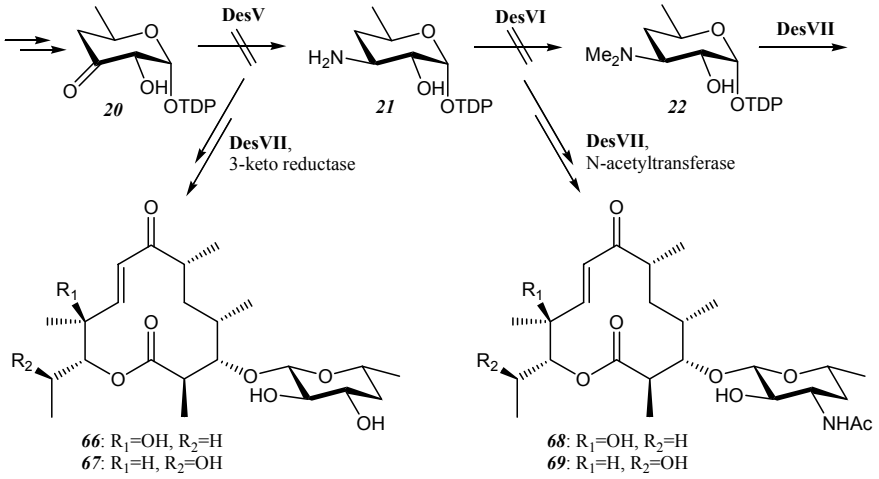

Figure 1-18: Novel macrolides produced by *S. venezuelae* mutants lacking DesV and DesVI activities.

in *S. venezuelae* that can stereospecifically reduce the C-3 keto group of the sugar metabolite. These results contradict an alternative pathway to desosamine favored at the time by Salah-Bey *et al.*, in which the introduction of the amino group was proposed to occur first at C-3 of the intermediate **23**, followed by C-4 deoxygenation.[94] Since the deletion of the *desV* gene leads to the incorporation of a 4-deoxy sugar in the final products, it is evident that deoxygenation at C-4 takes place prior to the transamination at C-3. Protein DesV was later overproduced in *E. coli* and purified to homogeneity.[163] The assay of DesV activity in the reverse direction, using a chemically synthesized compound **21** as substrate, led to the production of **20**. These experiments provide unequivocal evidence establishing the role of DesV as the aminotransferase required for the C-3 transamination step in the biosynthesis of desosamine.

The fermentation of the KdesVI mutant resulted in the isolation of another pair of methymycin/neomethymycin analogs, **68** and **69**, each containing a 3,4,6-trideoxy-3-acetamido-D-glucose (Figure 1-18).[96] Since no *N*-methylated sugar derivatives were obtained in the absence of *desVI*, its encoded protein DesVI must be the desired *N*,*N*-dimethyltransferase catalyzing *N*-methylation in the desosamine biosynthesis. Interestingly, instead of the expected sugar with a free amino group, each of the fermentation products carried an acetylated amino hexose. It has been proposed that the observed *N*-acetylation might be a necessary step for the self-protection of *S. venezuelae*.[96] The biochemical evidence supporting the DesVI function assignment was obtained by using heterologously produced and homogeneously purified DesVI in the activity

assay.[99, 100] The isolation of product **22** from the incubation of compound **21**, SAM, and purified DesVI confirms the function of DesVI as a *N*,*N*-dimethyl-transferase.

These gene disruption experiments demonstrated the feasibility of such an approach for the investigation of the deoxysugar biosynthetic pathway. The results also provided strong evidence for the relaxed substrate specificity of the glycosyltransferase DesVII based on its ability to accept various unnatural sugar substrates. Moreover, these studies resulted in the production of a series of "unnatural" natural products, setting up the stage for future application of the gene disruption technique towards the creation of new macrolide antibiotics.[137]

The last step of the macrolide biosynthesis in *S. venezuelae* is the hydroxylation at C-10 and C-12 of **3**, giving rise to **1** and **2**, respectively, and also at C-12 of **5**, resulting in **4** (Figure 1-19). Initial studies demonstrated that these

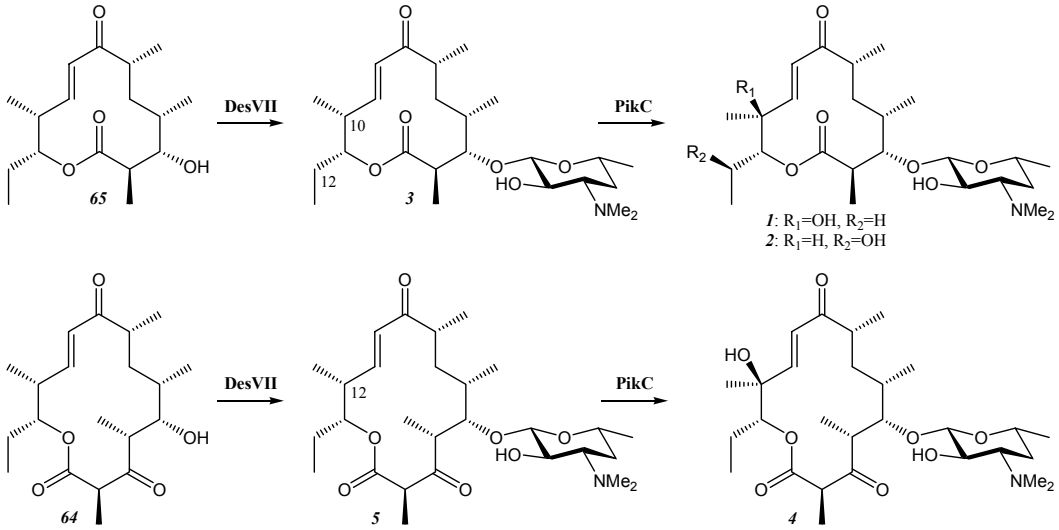

Figure 1-19: Final steps of macrolide biosynthesis by *S. venezuelae*.

hydroxylations are catalyzed by a cytochrome P450 monooxygenase (or monooxygenases), and are preceded by the glycosylation of **64** and **65**.[164, 165] The sequencing of the pikromycin gene cluster[95] and the independent screening of the *S. venezuelae* genome[165] have identified a single gene encoding such a P450 enzyme, *pikC* (also referred to as *picK*). PikC has been heterologously produced in *E. coli* and shown to be capable of catalyzing the hydroxylation of **3** and **5** *in vitro* with a slight preference for **5** (k_{cat}/K_m: 0.023±0.01 vs. 0.008±0.004 $\mu M^{-1}s^{-1}$).[166] The inactivation of *pikC* in *S. venezuelae* abolished the production of all hydroxylated macrolides, 12- and 14-membered, and gave only **3** and **5** as the products.[167] These results confirmed that *S. venezuelae* possesses a single P450 enzyme, PikC, responsible for the hydroxylation of both **3** and **5**. It is worth mentioning, that macrolide-hydroxylating enzymes, *e.g.* EryF and EryK involved in the erythromycin biosynthesis, usually have a stringent substrate specificity.[118, 168] In contrast, PikC discriminates between glycosylated and unglycosylated macrolides, but is able to accept macrolides of two ring sizes and hydroxylate **3** at two different positions. These unusual properties may prove to be useful for a combinatorial approach to biosynthetically produce new macrolides.

In summary, the biosynthesis of macrolides by *S. venezuelae* has a number of unique features. It is the only example known where a single PKS naturally produces macrolides of two different ring sizes. Its post-PKS enzymes, DesVII and PikC, exhibit an unprecedented versatility in the modification of both 12- and 14-membered macrolactone rings. All of the macrolides produced by

S. venezuelae contain a single carbohydrate substituent, D-desosamine. This 3-amino-3,4,6-trideoxy sugar moiety is a common sugar component of clinically important macrolides. Its formation is intriguing because of the yet unknown mechanism of the C-4 deoxygenation step. Finally, genetic manipulations of *S. venezuelae* proved to be useful for the generation of novel metabolites, and its potential applications certainly worth further exploration.

1.6 THESIS STATEMENT

Combinatorial biosynthesis of secondary metabolites has been an emerging strategy for making novel compounds. Over the last ten years, the biosynthetic routes to several clinically important macrolide antibiotics have been extensively studied, and significant progress has been made in our understanding of the underlying chemistry of the key steps in each pathway. However, our knowledge of the biosynthesis of the deoxy sugar components in macrolides and their attachment to the respective aglycone remains scarce. The above overview of the biosynthesis of desosamine in *S. venezuelae* clearly shows that, more efforts are needed to fully elucidate the detailed pathway, especially the roles of *desI*, *desII* and *desVIII*.

Described in Chapter 2 of this dissertation are the experiments intended to uncover the details of the formation of desosamine in *S. venezuelae*, in particular, the results of the gene disruption analyses of *desI*, *desII*, and *desVIII*. Chapter 3 focuses on the study of the secondary self-protection mechanism in *S. venezuelae* and documents the discovery and the initial analysis of the glycosyltransferase DesG involved in the self-resistance to macrolides. The last chapter is dedicated

to an in-depth study of the glycosyltransferase DesVII, which catalyzes the coupling of desosamine to the aglycones. The *in vitro* reconstitution of the DesVII activity proved to be difficult, but the problem was eventually resolved because of the identification of its catalytic partner, DesVIII, as an essential component for the glycosyltransferase activity. The study has resulted in the first report of a demonstrated macrolide glycosyltransferase activity *in vitro*.

As a whole, the work presented here contributes significantly to our understanding of the biosynthesis of macrolide antibiotics, specifically the formation of a novel deoxy hexose and its coupling to the macrolactone aglycone. The mechanisms of an unusual self-resistance were also biochemically established. Moreover, the discovery of the specific requirement of a second protein component for the glycosyl transfer activity of DesVII opens new possibilities for future *in vitro* generation of biologically active macrolides using unnatural aglycones and various sugar nucleotides.

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Chapter 2: Probing Biosynthetic Steps and Enzyme Activities of D-Desosamine Biosynthesis Using Gene Disruption Experiments

2.1 INTRODUCTION

D-desosamine is a common sugar component found in many macrolide antibiotics. The biosynthesis of TDP-D-desosamine (**22**) has attracted considerable attention from several research groups.[1-5] Extensive genetic and biochemical studies of the pathway to **22** in *S. erythraea*[6-8] and in *S. venezuelae*[9-12] have been performed. However, parts of the proposed biosynthetic scheme for the formation of **22** (Figure 1-17) remain speculative. In particular, the functions of the *desI*, *desII*, and *desVIII* genes (and their homologues in the erythromycin pathway) are assigned solely on the basis of sequence homology to other known genes.

The biosynthesis of **22** in the producer of macrolide antibiotics **1**, **2**, **4**, and **5**, *S. venezuelae*, represents a particularly attractive system suitable for genetic studies for several reasons. Firstly, D-desosamine is the sole carbohydrate residue in these natural products thus simplifying the genetic manipulations to be carried out as well as the structural analysis of the secondary metabolites produced by these mutant strains. Secondly, growth conditions for the culture of *S. venezuelae*[13] in addition to procedures for the genetic alteration of this particular species have been established.[14, 15] Lastly, the glycosyltransferase DesVII involved in the biosynthesis of **1**, **2**, **4**, and **5** has been shown to possess a

rather relaxed substrate specificity.[9, 10] This last property is desirable for the construction of hybrid metabolites using genetic engineering.

Gene disruption is a useful tool in the study of natural product biosynthesis. A correlation between the generated mutant and its phenotype (accumulated intermediates) can be helpful in assigning the function of the gene as well as in establishing the biosynthetic pathway. The biosynthesis of macrolides is composed of two pathways that are connected through the action of glycosyltransferases: the macrolactone synthesis by the PKS, and the deoxy sugar nucleotide synthesis by a set of dedicated sugar biosynthetic genes. The macrolactone derivatives generated by the manipulation of the PKS genes can be readily isolated from the fermentation culture of the mutants through organic solvent extraction. However, the detection and/or isolation of the TDP-sugar intermediates accumulated in the macrolide producing strains can be difficult due to their instability and the high water solubility. Only those sugar intermediates that are recognized and coupled to the aglycone by the existing glycosyl-transferase can be isolated as a part of the macrolide products. Thus, the interpretation of the disruption studies of deoxy sugar genes should be taken with caution, since the results may not provide a direct correlation between gene function and the biosynthetic pathway. Insight can only be obtained if the glycosyltransferase can incorporate the accumulated sugar intermediate into the final compound in sufficient amounts for isolation and characterization.

This chapter summarizes the experiments designed to investigate the roles of the *desI*, *desII* and *desVIII* genes in the biosynthesis of **22**, using gene

disruption and gene replacement methods. Our goals are to establish the biosynthetic pathway to **22** and to evaluate the potential of a combinatorial biosynthetic approach towards the generation of novel macrolide antibiotics containing unusual sugar appendages *via* genetic manipulation of the biosynthetic machinery.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 General

Bacterial Strains. *E. coli* DH5α from Invitrogen (Carlsbad, CA) was used throughout the study as the regular cloning host. *E. coli* S17-1[16] was the donor strain for the conjugal transfer to *S. venezuelae*. Strains *S. venezuelae* ATCC 15439, *Streptomyces peucetius* ATCC 29050, and *S. erythraea* ATCC 11635 were obtained from American Type Culture Collection (ATCC, Rockville, MD) as freeze-dried pellets and were revived according to the instructions provided by ATCC.

Plasmids, Vectors and DNA Manipulations. Cosmid pLZ4 (a derivative of the pNJ1 vector)[17] containing the desosamine biosynthetic cluster and a part of the polyketide synthase cluster[14, 15] was used as the template for the polymerase chain reaction (PCR) to obtain the appropriate DNA fragments. Plasmid pL005 was used as the source of the appropriate DNA fragments for the *desII* disruption construct.[15] Plasmid pKC1139, used for the conjugal transfer of DNA to *S. venezuelae*, was a gift from Dr. Leonard Katz of Abbott Laboratories.[18] Vector pDHS617, used for the complementation of the *S. venezuelae* mutant strains with the additional genes, was a gift from Professor David Sherman of the University

of Michigan.[19] Cosmid pFD666 was used as the source of the *neo* fragment.[20, 21] Cosmid pZHG4 was used as the template for the PCR amplification of the *tylM3* gene of *S. fradiae*.[22] The general methods and protocols for recombinant DNA manipulations were followed per the description by Sambrook *et al.*,[23] and those dealing with *Streptomyces* strains per the description by Hopwood *et al.*[24] and Kieser *et al.*[25]

Biochemicals. The enzymes used in the cloning experiments were obtained from Invitrogen or Promega (Madison, WI). The ^{32}P labeled nucleotides and the Multiprime DNA Labeling System used for the DNA probe labeling during the Southern blot hybridization analysis were purchased from Amersham Biosciences (Piscataway, NJ). Antibiotics and biochemicals used in this study were products of Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The growth media components were obtained from BD Diagnostics System (Sparks, MD). The purification of DNA after digestion reactions and from an agarose gel was achieved using the Bio101 Geneclean II® kit purchased from Fisher Scientific.

Instrumentation. The pH values were obtained using a Corning pH meter 240 purchased from Fisher Scientific. The mini-sub cell GT from Biorad (Richmond, CA), powered by either a FB600 or a FB135 power supply from Fisher Scientific, was used for agarose gel electrophoresis. All centrifugation procedures were performed using an Avanti J-25 unit from Beckman (Arlington Heights, IL). Microcentrifugations were done with an Eppendorf 5415C from Brinkmann Instruments, Inc (Westbury, NY). The gel documentation was

performed with a Polaroid MP-4 Land Camera (Cambridge, MA) loaded with a Polaroid Type 667 coatless black and white instant film, and illuminated with a Foto UV 310 transilluminator from Fotodyne Inc (New Berlin, WI). The polymerase chain reactions were conducted with a Perkin Elmer Cetus DNA Thermal Cycler (Norwalk, CT). The HPLC separations were achieved on a Beckman 366 instrument (Beckman Instruments, Fullerton, CA) equipped with Econosil C_{18} columns from Alltech (Deerfield, IL). The NMR spectra were acquired on a Varian Unity 300 or 500 spectrometer, and the chemical shifts (δ in ppm) are given relative to those for Me_4Si (for ^1H and ^{13}C) with the coupling constants reported in hertz (Hz). The high-resolution FAB mass spectra were obtained using a VG 7070E-HF mass spectrometer. Flash chromatography was performed on Lagand Chemical silica gel (230-400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm) (Macherey-Nagel Inc., Easton, PA).

Preparation of Competent Cells. Cells were made competent according to the RbCl method.[23] A single fresh colony of the appropriate *E. coli* strain was used to inoculate 2 mL of the Luria-Bertani (LB) liquid medium and the resulting culture was grown overnight at 37 °C with shaking at 250 rpm. A 500 μL aliquot of an overnight culture was used to inoculate 50 mL of the LB medium in an Erlenmeyer flask. When the cell growth at 37 °C reached an OD_{600} of approximately 0.4, the culture was transferred into a pre-chilled polypropylene tube and incubated on ice for 30 min. After centrifugation at 3,000 *g* for 5 min, the supernatant was discarded, and the cell pellet was gently resuspended in

13 mL of an ice-cold RF1 solution (100 mM RbCl, 15% glycerol, 50 mM $MnCl_2$, 30 mM potassium acetate, 10 mM $CaCl_2$, pH 5.8, sterilized by filtering through a 0.22 μm membrane). After incubation on ice for 15 min, another centrifugation step at 3,000 *g*, 4 °C for 5 min was performed and the resulting cell pellet was resuspended in 4 mL of an ice-cold RF2 solution (10 mM RbCl, 10 mM MOPS, 75 mM $CaCl_2$, 15% glycerol, pH 6.8). The cells were aliquotted into 100 μL portions in pre-chilled microcentrifuge tubes and frozen at –80 °C.

PCR Amplification of DNA. The general procedure for the polymerase chain reaction amplification of DNA fragments was followed as described below. Two oligonucleotide primers complementary to the sequences at each end of the target gene were designed and the appropriate restriction enzyme sites were incorporated when necessary. A polymerase-mediated amplification was carried out in a 0.5 mL microcentrifuge tube. The reaction mixture consisted of 59.5 μL of de-ionized water, 10 μL of the *pfu* polymerase buffer (10x), 8 μL of the deoxy-ribonucleotidyl triphosphate mix (2.5 mM each), 10.0 μL of DMSO, 2.5 μL of 40% glycerol, 4 μL of each of the primers (20 μM stock concentration), 1.0 μL of the template (approximately 0.1 μg), and 1.0 μL of the cloned *pfu* polymerase (2.5 units, Invitrogen). The reaction mixture was overlaid with a drop of mineral oil and subjected to the following thermal cycling: (1) 1 cycle of incubation at 95 °C for 3 min (2) 30 cycles of incubation at 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 90 s. The tubes were held at 4 °C prior to being removed from the thermal cycler.

2.2.2 Construction of the Disruption Plasmids

Plasmid pDesI-K. Plasmid pDesI-K (Figure 2-1) was generated for the deletional inactivation of the *desI* gene in *S. venezuelae*. For the construction of pDesI-K, a 1.1 kb fragment of *desII* located downstream of *desI*, "desI", was amplified by PCR using pLZ4 as the template with the following primers: 5′-GCGCGAATTCGCTCCTCGAAGGCGTTGAGG-3′ and 5′-GCGCTCTAGA-CATCCACGCCCACGATTGG-3′ (the engineered restriction sites *Eco*RI and *Xba*I, respectively, are underlined). A 1.0 kb fragment located upstream of *desI*, "sug-d", was amplified by PCR using the same template and the following primers: 5′-GGCCAAGCTTGGAGTCGATGAGC-3′ and 5′-GGCCCTGCAG-AAGAATGCGAGGTCG-3′ (the engineered restriction sites *Hind*III and *Pst*I, respectively, are underlined). The fragment containing the neomycin resistance gene ("neo", 1.4 kb) was amplified by PCR using pFD666 as the template with primers 5′-CGCGTCTAGATACCTACAGCGTGAGC-3′ and 5′-CGCG-CTGCAGCCACGAATTAGCC-3′ containing the restriction sites *Xba*I and *Pst*I (underlined), respectively.

The three fragments, "desI", "sug-d", and "neo", were digested with the appropriate restriction enzymes and simultaneously ligated into the *Hind*III-*Eco*RI sites of pUC119. The 3.5 kb insert of the resulting plasmid pDesI-d1 was then removed by the digestion with *Eco*RI-*Hind*III and cloned into the same restriction sites of the pKC1139 vector, resulting in the plasmid pDesI-K (Figure 2-1).

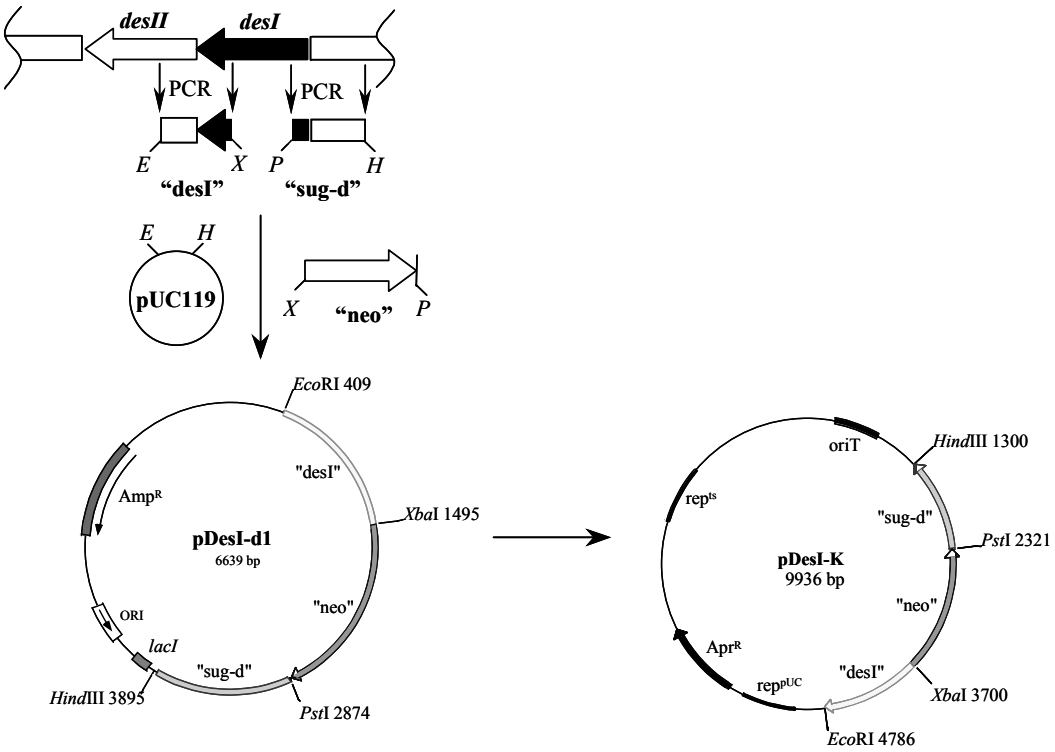


Figure 2-1: Construction of the disruption plasmid pDesI-K. The endonuclease restriction sites are abbreviated as follows: *E*, *Eco*RI; *H*, *Hind*III; *P*, *Pst*I; *X*, *Xba*I. Plasmid pDesI-d1 is derived from pUC119 and contains fragments "desI", "neo", and "sug-d". The origin of DNA replication (ORI), *lacI* operon, and the ampicillin resistance gene (Amp^R) of pUC119 are shown. Plasmid pDesI-K is derived from pKC1139 and contains the *E. coli* origin of DNA replication from the pUC plasmid (rep^{pUC}), the apramycin resistance gene (Apr^R), a temperature sensitive replicon from *Streptomyces ghanaensis* functioning only at temperatures below 34 ^oC (rep^{ts}), and the origin of transfer for conjugation (oriT).

Construction of Plasmid pDesII-K. Plasmid pDesII-K (Figure 2-2) was generated for the deletional inactivation of the *desII* gene in *S. venezuelae*. The 1.2 kb fragment "desII-up" located upstream of *desII* was amplified by PCR using pLZ4 as the template with the following primers: 5′-CGCGCTGCAGTCCAAT-

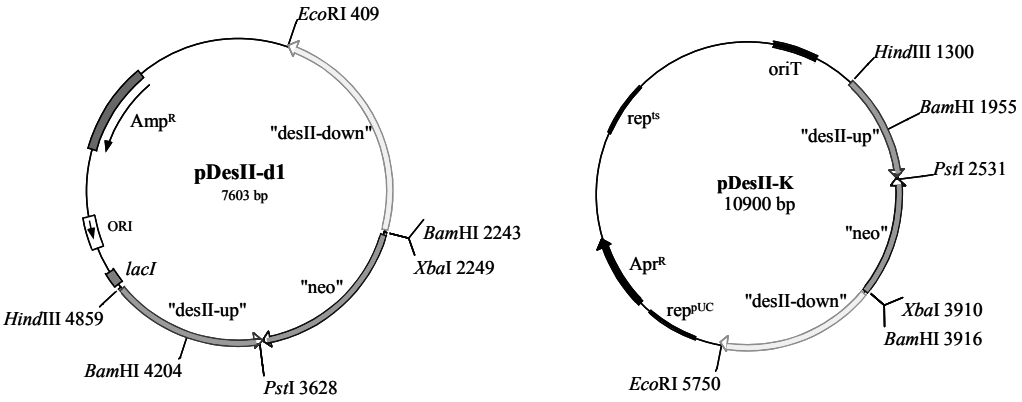


Figure 2-2: Construction of the disruption plasmid pDesII-K. Abbreviations are as described in the caption to Figure 2-1.

CGTGGGCGTGGATGTC-3′ and 5′-CGCGAAGCTTATCCGACCTCGCATTC-3′ (the engineered restriction sites *Pst*I and *Hind*III, respectively, are underlined). To obtain the fragment "desII-down" located downstream of *desII*, the plasmid pL005 was digested with the restriction endonuclease *Sma*I first. A 2.2 kb portion of the digested DNA was purified from the agarose gel and cloned at the *Pvu*II site of pUC119 through the blunt-ended ligation. The resulting plasmid pDesII-d2 was digested with the *Hind*III and *Xba*I enzymes. It was then ligated with the fragments "neo" and "desII-up" producing the plasmid pDesII-d1. The 4.5 kb *Eco*RI-*Hind*III fragment of pDesII-d1 was subsequently cloned into the same restriction sites of pKC1139 giving pDesII-K (Figure 2-2).

Construction of the Plasmid pDesVIII-K. Plasmid pDesVIII-K (Figure 2-3) was generated for the deletional inactivation of the *desVIII* gene in *S. venezuelae*. A strategy similar to the one described for the construction of pDesI-K was used to generate pDesVIII-K. Two fragments, "sug-up" (0.9 kb) and "desVIII" (a part of the *desVII* gene, 1.1 kb), flanking *desVIII* upstream and

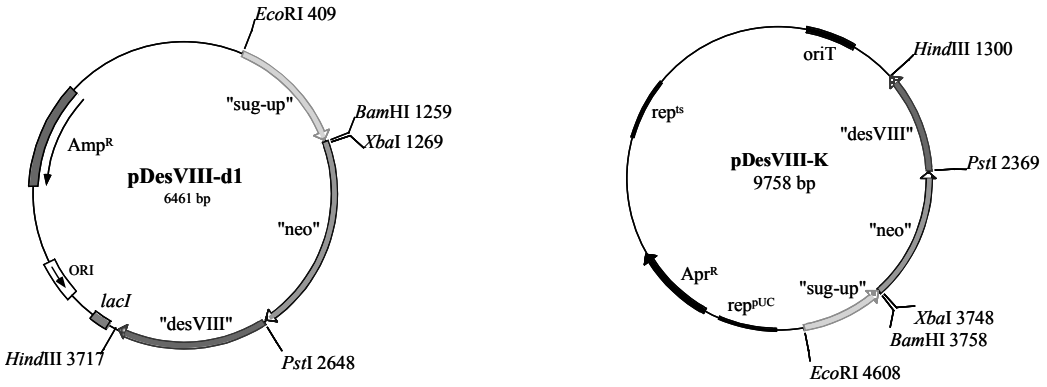


Figure 2-3: Construction of the disruption plasmid pDesVIII-K. Abbreviations are as described in the caption to Figure 2-1.

downstream, respectively, were amplified by PCR using pLZ4 as the template. Primers 5′-CCGGTCTAGACGTGGATCCAGTGGATGC-3′ and 5′-CGCG-GAATTCCAGCTACTTCTTCC-3′ were used for the amplification of the "sug-up" fragment (the engineered restriction sites *Xba*I and *Eco*RI, respectively, are underlined) and 5′-GCGCAAGCTTGGTGTGCATCGGGTAGTTGC-3′ and 5′-CCGGCTGCAGTCAGCAGCTCCTGAGACAC-3′ were used for the amplification of the "desVIII" fragment (the engineered restriction sites *Hind*III and *Pst*I, respectively, are underlined). These two fragments and the "neo" fragment were ligated into pUC119 to give the plasmid pDesVIII-d1. The 3.3 kb insert of pDesVIII-d1 was then removed by digestion with restriction enzymes *Eco*RI and *Hind*III and cloned into pKC1139, resulting in the plasmid pDesVIII-K (Figure 2-3).

2.2.3 Conjugal Transfer of Disruption Plasmids into *S. venezuelae*

These experiments were performed by following a published procedure[18] with minor modifications. Plasmids pDesI-K, pDesII-K, and pDesVIII-K were

initially propagated in *E. coli* DH5α but were subsequently transferred to *E. coli* S17-1 prior to being transferred to *S. venezuelae*. All of the culture tubes and flasks were equipped with glass beads or springs.

A 1 mL aliquot of *S. venezuelae* (recipient) mycelia was inoculated into 9 mL of the tryptic soy broth (TSB) and grown at 29 ^{o}C for 18 h. Meanwhile, *E. coli* S17-1, which contains the disruption plasmid (donor), was streaked out on LB plates containing apramycin (100 μg/mL), kanamycin (50 μg/mL), and streptomycin (10 μg/mL) and incubated at 37 ^{o}C overnight. Subsequently, an aliquot (2 mL) of the recipient culture was re-inoculated into 18 mL of TSB and allowed to grow for another 18 h. A brief sonication was carried out to homogenize the culture when cells were forming clumps. Single colonies of donor cells were inoculated into 2 mL of TSB containing apramycin (100 μg/mL), kanamycin (50 μg/mL), and streptomycin (10 μg/mL), and allowed to grow at 37 ^{o}C overnight in an orbital shaker. Afterwards, 1 mL of the recipient culture was transferred into 9 mL of TSB for a 3 h growth. In parallel, an overnight donor culture (20 μL) was transferred into 2 mL of TSB containing the appropriate antibiotics, and allowed to grow at 37 ^{o}C for 3 h.

Both recipient and donor cells were recovered by centrifugation and washed twice with TSB. A 2 mL aliquot of TSB was then used to resuspend the recipient and donor cells separately. Three mixtures of the recipient and donor cells were prepared with ratios of 1:1, 9:1, and 1:9, respectively. Aliquots of 100 μL of the mixed cultures were spread on freshly prepared AS1 agar plates (per 1 L: 1 g of yeast extract, 0.2 g of L-alanine, 0.5 g of L-arginine, 5 g of soluble

starch, 2.5 g of NaCl, 10 g of Na_2SO_4, 20 g of agar, pH 7.5; after autoclaving, $MgCl_2$ was added to the final concentration of 10 mM).[26] Three to five replicates were normally made for each mixture. In addition, two plates were inoculated with *Streptomyces* cells and one plate was inoculated with *E. coli* cells as controls. The plates were incubated at 29 ^{o}C overnight prior to overlaying with the appropriate antibiotics. For conjugation plates, 1 mL of aqueous solution of nalidixic acid (500 μg/mL), apramycin (500 μg/mL) and kanamycin (500 μg/mL) was applied on the surface. For negative controls, one *Streptomyces* plate was overlaid with all three antibiotics, and one *E. coli* plate was overlaid with nalidixic acid (500 μg/mL). For a positive control, one *Streptomyces* plate was overlaid with nalidixic acid (500 μg/mL). The plates were incubated at 29 ^{o}C for 7 to 10 days.

2.2.4 Screening for Double-Crossover Mutants

Plasmids pDesI-K, pDesII-K, and pDesVIII-K were introduced into the wild type *S. venezuelae via* conjugal transfer. The conjugants usually became visible on the plates after 4–5 days of incubation. Individual colonies were picked and streaked on SPA plates (per 1 L: 1 g of yeast extract, 1 g of beef extract, 2 g of tryptone, 10 g of glucose, several crystals of $FeSO_4$, 15 g of agar) supplemented with apramycin (100 μg/mL) and kanamycin (50 μg/mL). Three spore-to-spore passages were carried out on SPA plates without antibiotics to facilitate the homologous recombination between the disruption plasmid and the *S. venezuelae* chromosome, and the natural loss of the plasmid by *S. venezuelae*. The resulting spores were inoculated into 5 mL of SGGP medium (per 1 L: 4 g of

peptone, 4 g of yeast extract, 4 g of casamino acids, 2 g of glycine, 0.25 g of $MgSO_4 \bullet 7\ H_2O$; after autoclaving, glucose was added to 1% w/v and KH_2PO_4 to the final concentration of 10 mM) without antibiotics for the growth at 29 ^oC. Overnight cultures were diluted 10^{-5}–10^{-7} fold with SGGP and plated on SPA supplemented with 50 μg/mL of kanamycin. Single colonies were then replicated on SPA plates containing 100 μg/mL of apramycin. The colonies, which showed kanamycin resistant (Kan^R) and apramycin sensitive (Apr^S) characteristics, were identified as double-crossover mutants. These mutant strains are referred to as KdesI, KdesII, and KdesVIII.

2.2.5 Preparation of Spore Suspensions and Frozen Mycelia for *S. venezuelae* Strains

The wild type *S. venezuelae* and its mutant strains were stored as spore suspensions in 20% glycerol at –80 ^oC. Colonies of *S. venezuelae* strains were first streaked out on SPA plates containing the appropriate antibiotics and allowed to grow for up to one week at 27–29 °C. To prepare spore suspensions, 2 mL of sterile 20% glycerol in water was added to each plate, and the spores were scraped off with small sterile Q-tips. The resulting suspensions were transferred to a pipette tip plugged with cotton wool. The spore suspensions were pipetted through cotton into sterile Eppendorf tubes and stored at –80 °C.

To prepare frozen mycelia of *S. venezuelae* strains, a 10 μL aliquot of spore suspension was inoculated into 25 mL of SGGP or TSB medium in a 250 mL baffled flask and allowed to grow for 28 h at 29 °C in an orbital shaker. Cell cultures were transferred to a 50 mL conical tube and centrifuged for 10 min at 1,700 *g*. The supernatant was discarded and cell pellet was resuspended in

15 mL of 10.3% sucrose. Cells were centrifuged again and the supernatant was discarded. Resuspension of cells in 15 mL of 20% glycerol yielded a stock mycelia solution that was stored at –20 °C.

2.2.6 Confirmation of Deletional Disruption in KdesII-101 Mutant by Southern Blot Hybridization

The replacement of the *desII* gene with the kanamycin resistance marker, *neo*, in the chromosome of one of the *S. venezuelae* KdesI mutants, KdesI-101, was confirmed by the Southern blot hybridization analysis. The samples of genomic DNA from the wild type *S. venezuelae* and KdesI-101 strains were prepared using a previously described procedure.[24] The genomic DNA was digested with the *Bam*HI enzyme, separated by agarose gel electrophoresis, and transferred to a nylon membrane using a standard procedure.[23] A 0.95 kb "desII-probe" fragment from the *desIII* region of the *S. venezuelae* chromosome and the *neo* fragment were used as probes. The fragment "desII-probe" was synthesized by PCR using the following primers: 5'-CGCGTCTTCCTTTCGGTCGC-GCTGTA-3' and 5'-GCGCTCGGTCATTTCGAAGCAGAT-3'. The probes were labeled with ^{32}P-containing nucleotides using the Multiprime DNA Labeling System (Amersham Biosciences) according to the manufacturer's instructions. Standard Southern hybridization protocols were used per description by Hopwood and coworkers.[24]

2.2.7 Purification and Characterization of Metabolites Produced by *S. venezuelae* KdesI, KdesII, and KdesVIII Mutants

General Procedure. One of each kind of *S. venezuelae* double-crossover mutant was selected for further experiments: KdesI-80, KdesII-101, and

KdesVIII-92. The analysis was carried out by growing the KdesI-80, KdesII-101, and KdesVIII-92 mutants under the conditions described by Cane *et al.*, favoring the formation of 12-membered ring macrolides (**1** and **2**).[13] Specifically, 5 mL of the seed medium (per 1 L: 20 g of glucose, 15 g of soybean flour, 5 g of $CaCO_3$, 1 g of NaCl, 0.002 g of $CoCl_2 \cdot 6H_2O$, pH 7.2) was inoculated with 10 μL of spore suspension of either KdesI-80, KdesII-101, or KdesVIII-92 mutant and grown at 29 ^oC for 48 h. The culture was then transferred to 50 mL of the vegetative medium (per 1 L: 20 g of glucose, 30 g of soybean flour, 2.5 g of $CaCO_3$, 1 g of NaCl, 0.002 g of $CoCl_2 \cdot 6H_2O$, pH 7.2) and allowed to grow for another 48 h. The mycelia and cellular debris were removed by centrifugation at 10,000 *g* for 20 min. The supernatant was collected, adjusted to pH 9.5 with 10 N KOH, and extracted four times with an equal volume of chloroform. The evaporation of the organic solvent resulted in a dark-yellow residue, which was analyzed by TLC employing a solvent mixture of chloroform/methanol/25% NH_4OH (90:9.9:0.1). Visualization of the compounds present in the crude extract was accomplished by exposure to vanillin stain (0.75% vanilla, 1.5% H_2SO_4, in methanol) followed by heating with the heat gun. Methymycin (**1**) and its derivatives showed a green color, whereas neomethymycin (**2**) and its derivatives showed an orange color.

To isolate the glycosylated methymycin/neomethymycin analogs, a large-scale fermentation was carried out in which 200 mL of the seed medium was inoculated with 20 μL of the spore suspension of the corresponding *S. venezueale* mutant strain. This mixture, after incubating at 29 ^oC for 48 h, was used to inoculate the vegetative medium (20 mL per liter). The crude products were first

purified by flash chromatography on silica gel using a gradient of 0–40% methanol in chloroform. Further purification was performed by HPLC on a C_{18} column (Econosil, 10×250 mm, 10 μm) eluted isocratically with 40% acetonitrile in water. The detector was set at 235 nm. Details related to each of the mutants are as follows.

Characterization of Metabolites Produced by S. venezuelae KdesI-80. The macrolides produced by the *desI*-deleted mutant of *S. venezuelae* were isolated from 8 L of the fermentation broth of KdesI-80. A total of 600 mg of 10-deoxymethynolide (**65**), 40 mg of methynolide (**74**), 2 mg of neomethynolide (**75**), and 3.2 mg of **76** were obtained.

^{1}H NMR (500 MHz, $CDCl_3$) of **65**: δ (ppm) 6.75 (1H, dd, J = 15.5, 5.5 Hz, 9-H), 6.42 (1H, dd, J = 15.5, 1.0 Hz, 8-H), 5.00 (1H, ddd, J = 8.2, 5.5, 2.2 Hz, 11-H), 3.56 (1H, dd, J = 10.2, 5.2 Hz, 3-H), 2.64 (1H, m, 10-H), 2.60 (1H, dq, J = 10.5, 7.0 Hz, 2-H), 2.53 (1H, m, 6-H), 1.70 (1H, m, 12a-H), 1.66 (1H, m, 5a-H), 1.57 (1H, m, 12b-H), 1.56 (1H, m, 5b-H), 1.31 (3H, d, J = 6.5 Hz, 2-Me), 1.28 (1H, m, 4-H), 1.22 (3H, d, J = 7.0 Hz, 6-Me), 1.12 (3H, d, J = 7.0 Hz, 10-Me), 1.01 (3H, d, J = 6.5 Hz, 4-Me), 0.92 (3H, t, J = 7.5 Hz, 12-Me).

^{1}H NMR (500 MHz, $CDCl_3$) of **74**: δ (ppm) 6.59 (1H, d, J = 15.8 Hz, 9-H), 6.34 (1H, d, J = 15.8 Hz, 8-H), 4.78 (1H, dd, J = 11.0, 2.0 Hz, 11-H), 3.58 (1H, d, J = 10.5 Hz, 3-H), 2.61 (1H, dq, J = 10.5, 7.0 Hz, 2-H), 2.54 (1H, m, 6-H), 1.94 (1H, ddq, J = 14.5, 7.5, 2.0 Hz, 12a-H), 1.63 (2H, t, J = 7.5 Hz, 5H), 1.52 (1H, ddq, J = 14.5, 11.0, 7.5 Hz, 12b-H), 1.37 (3H, s, 10-Me), 1.33 (3H, d, J =

7.5 Hz, 2-Me), 1.30 (1H, m, 4-H), 1.21 (3H, d, J = 7.0 Hz, 6-Me), 1.09 (3H, d, J = 6.0 Hz, 4-Me), 0.91 (3H, t, J = 7.2 Hz, 12-Me).

^{1}H NMR (500 MHz, $CDCl_3$) of **75**: δ (ppm) 6.63 (1H, dd, J = 16.0, 5.5 Hz, 9-H), 6.44 (1H, dd, J = 16.0, 1.5 Hz, 8-H), 4.84 (1H, dd, J = 9.0, 2.2 Hz, 11-H), 3.90 (1H, dq, J = 5.5, 3.0 Hz, 12-H), 3.57 (1H, d, J = 10.5 Hz, 3-H), 2.61 (1H, dq, J = 10.5, 7.0 Hz, 2-H), 2.44 (1H, m, 6-H), 1.62 (1H, t, J = 12.5 Hz, 5a-H), 1.45 (1H, dd, J = 12.5, 5.5 Hz, 5b-H), 1.31 (3H, d, J = 7.0 Hz, 2-Me), 1.31 (1H, m, 4-H), 1.22 (3H, d, J = 7.0 Hz, 6-Me), 1.22 (3H, d, J = 6.5 Hz, 12-Me), 1.18 (3H, d, J = 7.0 Hz, 10-Me), 1.02 (3H, d, J = 6.5 Hz, 4-Me).

^{1}H NMR (500 MHz, $CDCl_3$) of **76**: δ (ppm) 6.76 (1H, dd, J = 16.0, 5.5 Hz, 9-H), 6.43 (1H, d, J = 16.0 Hz, 8-H), 4.97 (1H, ddd, J = 8.4, 5.9, 2.5 Hz, 11-H), 4.29 (1H, d, J = 8.0 Hz, 1'-H), 3.62 (1H, d, J = 10.5 Hz, 3-H), 3.49 (1H, t, J = 9.0 Hz, 3'-H), 3.36 (1H, dd, J = 9.0, 8.0 Hz, 2'-H), 3.32 (1H, dq, J = 8.5, 5.5 Hz, 5'-H), 3.23 (1H, dd, J = 9.0, 8.5 Hz, 4'-H), 2.82 (1H, dq, J = 10.5, 7.0 Hz, 2-H), 2.64 (1H, m, 10-H), 2.55 (1H, m, 6-H), 1.70 (1H, m, 12a-H), 1.66 (1H, bt, J = 12.5 Hz, 5b-H), 1.56 (1H, m, 12b-H), 1.40 (1H, dd, J = 12.5, 4.5 Hz, 5a-H), 1.35 (3H, d, J = 7.0 Hz, 2-Me), 1.31 (3H, d, J = 5.5 Hz, 5'-Me), 1.24 (1H, bdd, J = 10.0, 4.5 Hz, 4-H), 1.21 (3H, d, J = 7.0 Hz, 6-Me), 1.11 (3H, d, J = 6.5 Hz, 10-Me), 1.00 (3H, d, J = 7.0 Hz, 4-Me), 0.92 (3H, t, J = 7.5 Hz, 12-Me). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 205.0 (C-7), 174.7 (C-1), 146.9 (C-9), 125.9 (C-8), 102.9 (C-1'), 85.4 (C-3), 76.5 (C-3'), 75.5 (C-4'), 74.7 (C-2'), 73.9 (C-11), 71.6 (C-5'), 45.0 (C-6), 43.9 (C-2), 37.9 (C-10), 34.1 (C-5), 33.4 (C-4), 25.2 (C-12), 17.7 (6-Me), 17.5 (5'-Me), 17.4 (4-Me), 16.2 (2-Me), 10.3 (12-Me), 9.6 (10-Me).

High-resolution (HR) FAB-MS: calculated for $C_{23}H_{38}O_8$ $(M + H)^+$ 443.2644, found 443.2661.

Characterization of Metabolites Produced by S. venezuelae KdesII-101. The macrolides produced by the *desII*-deleted mutant of *S. venezuelae* were isolated from 3 L of the fermentation broth of KdesII-101. Medium pressure liquid chromatography (MPLC) on silica gel with a gradient of 0–20% methanol in chloroform was used to separate the products. It was followed by further HPLC purification of fractions of interest as described above. A total of approximately 560 mg of 10-deoxymethynolide (**65**), approximately 80 mg of methynolide (**74**), approximately 20 mg of neomethynolide (**75**), 2.4 mg of **77** and 1 mg of **78** were obtained.

^{1}H NMR (500 MHz, $CDCl_3$) of **77**: δ(ppm) 6.75 (1H, dd, J = 16.0, 5.5 Hz, 9-H), 6.44 (1H, dd, J = 16.0, 1.2 Hz, 8-H), 5.34 (1H, d, J = 8.0 Hz, N-H), 4.96 (1H, m, 11-H), 4.27 (1H, d, J = 7.5 Hz, 1'-H), 3.66 (1H, dd, J = 9.5, 8.0 Hz, 4'-H), 3.60 (1H, d, J = 10.5 Hz, 3-H), 3.50 (1H, t, J = 9.5 Hz, 3'-H), 3.4 (1H, m, 5'-H), 3.4 (1H, m, 2'-H), 2.84 (1H, dq, J = 10.5, 7.0 Hz, 2-H), 2.64 (1H, m, 10-H), 2.53 (1H, m, 6-H), 2.06 (3H, s, **Me**-C=O), 1.7 (1H, m, 12a-H), 1.66 (1H, m, 5a-H), 1.56 (1H, m, 12b-H), 1.4 (1H, m, 5b-H), 1.36 (3H, d, J = 7.5 Hz, 2-Me), 1.25 (3H, d, J = 6.5 Hz, 6'-H), 1.24 (1H, m, 4-H), 1.21 (3H, d, J = 7.5 Hz, 6-Me), 1.10 (3H, d, J = 6.5 Hz, 10-Me), 0.99 (3H, d, J = 6.0 Hz, 4-Me), 0.91 (3H, t, J = 7.2 Hz, 12-Me). ^{13}C NMR (125 MHz, $CDCl_3$): δ(ppm) 205.3 (C-7), 175.1 (C-1), 171.9 (Me-**C=O**), 147.1 (C-9), 126.1 (C-8), 103.0 (C-1'), 85.8 (C-3), 75.8 (C-5'), 75.8 (C-3'), 74.1 (C-11), 70.8 (C-2'), 57.6 (C-4'), 45.3 (C-6), 44.0 (C-2), 38.1 (C-10), 34.2 (C-

5), 33.6 (C-4), 25.4 (C-12), 23.7 (**Me**-C=O), 18.1 (C-6'), 17.9 (6-Me), 17.6 (4-Me), 16.4 (2-Me), 10.5 (12-Me), 9.8 (10-Me). HR FAB-MS: calculated for $C_{25}H_{42}O_8N$ $(M + H)^+$ 484.2910, found 484.2904.

^{1}H NMR (500 MHz, $CDCl_3$) of **78**: δ (ppm) 6.69 (1H, dd, J = 16.0, 6.0 Hz, 11-H), 6.09 (1H, dd, J = 16.0, 1.5 Hz, 10-H), 5.35 (1H, d, J = 8.5 Hz, N-H), 4.96 (1H, m, 13-H), 4.36 (1H, d, J = 7.5 Hz, 1'-H), 4.19 (1H, m, 5-H), 3.83 (1H, q, J = 7.0 Hz, 2-H), 3.68 (1H, dt, J = 10.0, 8.5 Hz, 4'-H), 3.52 (1H, t, J = 8.5 Hz, 3'-H), 3.50 (1H, m, 5'-H), 3.42 (1H, t, J = 8.5 Hz, 2'-H), 2.92 (1H, dq, J = 7.5, 5.0 Hz, 4-H), 2.81 (1H, m, 8-H), 2.73 (1H, m, 12-H), 2.06 (3H, s, **Me**-C=O), 1.8 (1H, m, 6-H), 1.6 (1H, m, 14a-H overlapping with H_2O peak), 1.55 (1H, m, 7a-H), 1.37 (3H, d, J = 6.5 Hz, 2-Me), 1.32 (3H, d, J = 7.0 Hz, 4-Me), 1.3 (1H, m, 14b-H), 1.27 (3H, d, J = 6.5 Hz, 6'-H), 1.25 (1H, m, 7b-H), 1.12 (3H, d, J = 6.0 Hz, 8-Me), 1.11 (3H, d, J = 6.5 Hz, 12-Me), 1.07 (3H, d, J = 6.0 Hz, 6-Me), 0.91 (3H, t, J = 7.2 Hz, 14-Me). HR FAB-MS: calculated for $C_{28}H_{46}O_9N$ $(M + H)^+$ 540.3172, found 540.3203.

2.2.8 Construction of the Expression Plasmids

Plasmids pDesVII, pDesVIII, pTylM3, pEryCII, and pDnrQ were constructed for the expression of the *desVII*, *desVIII*, *tylM3*, *eryCII*, and *dnrQ* genes, respectively, in the *S. venezuelae* KdesVIII mutant. The fragments containing these genes were amplified by PCR using the primers listed in Table 2-1. These primers were designed to contain restriction sites for *Eco*RI (underlined in the forward primer) and *Xba*I (underlined in the reverse primer) for the subsequent cloning. One base in the sequence of the forward primer for the

Table 2-1: Primers Used for the Amplification of the Heterologous Genes[a]

Fragment	Size, kb	Primers (5′ to 3′)	Restriction Sites
"desVII"	1.3	GGCCGAATTCTAAGGAAGGACACGACG GCGCTCTAGATACAGGGGTGAG	*Eco*RI *Xba*I
"desVIII"	1.3	GGCCGAATTCTGAAGAGATGGCAGAAC GCGCTCTAGACACACGGGACGGTCTGA	*Eco*RI *Xba*I
"tylM3"	1.3	GGCCGAATTCTGAGAGGAGAGGCCGTGAACA CGCGTCTAGAGTGCCCTTCTCACT	*Eco*RI *Xba*I
"eryCII"	1.1	GGCCGAATTCTGAGGAGGGAAT[A]CATGACCAC GCGCTCTAGAGTTACCTCAGAGCTC	*Eco*RI *Xba*I
"dnrQ"	1.4	GGCCGAATTCTGAGGAGCACGACGATG GCGCTCTAGAGCACCTTCATGGGGTCAC	*Eco*RI *Xba*I

[a] The respective restriction sites are underlined in the sequence of the primers.

"eryCII" fragment was modified (T→A silent mutation of the residue shown in brackets) to eliminate the innate *Eco*RI restriction site. Cosmid pLZ4 was used as the template for the amplification of the "desVII" and "desVIII" fragments and the cosmid pZHG4 was used as the template for PCR amplification of the "tylM3" fragment. Genomic DNA of *S. erythraea* and *S. peucetius* digested with *Bam*HI was used as the template for the amplification of the fragments "eryCII" and "dnrQ", respectively. The PCR products were cloned into the *Eco*RI-*Xba*I sites of the vector pDHS617, containing the apramycin resistance marker, to give plasmids pDesVII (Figure 2-4), pDesVIII, pTylM3, pEryCII, and pDnrQ. Each of these plasmids was used to transform *E. coli* S17-1 and then introduced into the KdesVIII-92 mutant.

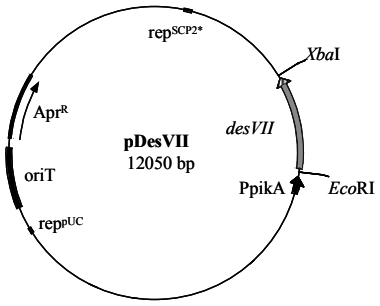


Figure 2-4: An example of the expression plasmid, pDesVII. Plasmids pDesVIII, pTylM3, pEryCII, and pDnrQ differ from pDesVII only by the sequence of the insert between the *Eco*RI and *Xba*I restriction sites. The promoter derived from the *S. venezuelae* (PpikAI), the streptomycete origin of DNA replication (rep^{SCP2*}), the apramycin resistance gene (Apr^R), the origin of transfer for conjugation (oriT), and the *E. coli* origin of DNA replication from the pUC plasmid (rep^{pUC}) are labeled.

2.2.9 Construction of *S. venezuelae* KdesVIII Strains Expressing *desVII*, *desVIII*, *tylM3*, *eryCII*, and *dnrQ* Genes

Each of the plasmids pDesVII, pDesVIII, pTylM3, pEryCII, and pDnrQ was introduced into the *S. venezuelae* KdesVIII-92 strain *via* conjugal transfer as described above. Selected colonies of *S. venezuelae* desVII/KdesVIII, desVIII/KdesVIII, tylM3/KdesVIII, eryCII/KdesVIII, and dnrQ/KdesVIII mutants were propagated on SPA plates containing apramycin (50 μg/mL) and kanamycin (50 μg/mL), and spore suspensions were prepared for storage.

2.2.10 Purification and Characterization of Metabolites Produced by *S. venezuelae* dnrQ/KdesVIII

The isolation and purification of the metabolites produced by the recombinant *S. venezuelae* dnrQ/KdesVIII strain were carried out as described above (Section 2.2.7). The crude products from 3 L of a vegetative culture

(yellow oil, approximately 120 mg) were first purified by flash chromatography on silica gel using a gradient of 0–24% methanol in chloroform. Further purification was performed by HPLC on a C_{18} column (Econosil, 10×250 mm, 10 μm) eluted isocratically with 40% acetonitrile in 57 mM ammonium acetate buffer (pH 6.7). A total of 40 mg of 10-deoxymethynolide (**65**), approximately 3 mg of each methymycin (**1**) and neomethymycin (**2**), approximately 4 mg of crude pikromycin (**4**), approximately 2–5 mg of crude methynolide (**74**) and neomethynolide (**75**), and approximately 7 mg of pure neomethymycin (**83**) were obtained.

^{1}H NMR (500 MHz, $CDCl_3$) of **83**: δ (ppm) 6.65 (1H, d, J = 16.0 Hz, 9-H), 6.31 (1H, d, J = 16.0 Hz, 8-H), 4.67 (1H, d, J = 9.0 Hz, 11-H), 4.29 (1H, d, J = 7.0 Hz, 1′-H), 4.13 (1H, dq, J = 9.0, 6.5 Hz, 12-H), 3.61 (1H, d, J = 10.5 Hz, 3-H), 3.52 (1H, dqd, J = 11.0, 6.5, 1.5 Hz, 5′-H), 3.32 (1H, dd, J = 10.5, 7.0 Hz, 2′-H), 2.88–2.80 (2H, m, 3′-H and 2-H), 2.57 (1H, m, 6-H), 2.44 (6H, s, -NMe_2), 1.76 (1H, ddd, J = 12.0, 3.5, 1.5 Hz, 4′eq-H), 1.67 (1H, bt, J = 13.5 Hz, 5a-H), 1.52 (3H, s, 10-Me), 1.44 (1H, m, 5b-H), 1.41 (3H, d, J = 6.5 Hz, 2-Me), 1.32 (1H, bq, J = 12.0 Hz, 4′ax-H), 1.25 (3H, d, J = 6.5 Hz, 5′-Me), 1.20 (1H, m, J = 6.5 Hz, 4-H), 1.184 (3H, d, J = 6.5 Hz, 12-Me), 1.178 (3H, d, J = 7.0 Hz, 6-Me), 1.01 (3H, d, J = 6.5 Hz, 4-Me). ^{13}C NMR (125 MHz, $CDCl_3$): δ (ppm) 204.1 (C-7), 174.1 (C-1), 148.3 (C-9), 125.4 (C-8), 104.7 (C-1′), 85.5 (C-3), 75.5 (C-10), 74.2 (C-11), 70.0 (C-2′), 68.9 (C-5′), 67.8 (C-12), 65.9 (C-3′), 45.2 (C-6), 44.0 (C-2), 39.8 (NMe_2), 33.8 (C-4), 33.7 (C-5), 29.1 (C-4′), 21.07 and 21.04 (5′-Me and 12-Me),

20.2 (10-Me), 17.6 (6-Me), 17.4 (4-Me), 15.9 (2-Me). HR FAB-MS: calculated for $C_{25}H_{43}NO_8$ $(M + H)^+$ 486.3067, found 486.3076.

2.3 RESULTS AND DISCUSSION

2.3.1 Inactivation of *desI via* Partial Gene Deletion

The protein encoded by *desI* shows sequence homology to coenzyme B_6-dependent enzymes and has been assigned as the dehydrase involved in the C-4 deoxygenation step. Hence, if *desI* is disrupted in the pathway, desosamine biosynthesis will be "terminated" prior to C-4 deoxygenation, leading to an accumulation of the 3-keto-6-deoxyhexose **23**, which may also be in equilibrium with the 4-keto intermediate **18** (Figure 2-5). As mentioned earlier, the production of **66** and **67** by the KdesV mutant is indicative of the existence of a pathway-independent reductase in *S. venezuelae* that could stereospecifically reduce the C-3 keto group of its sugar metabolite. It is possible that the same

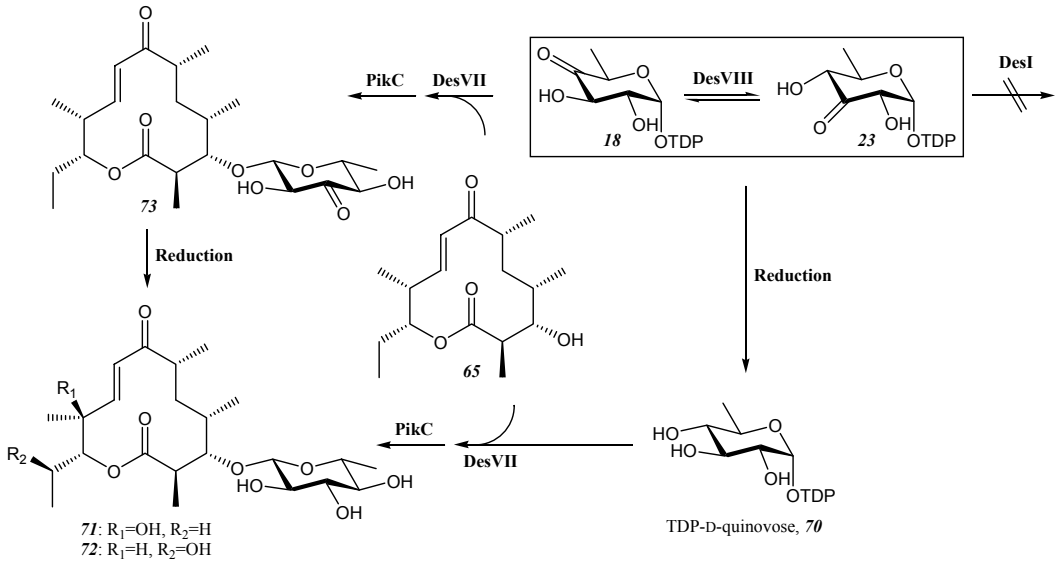


Figure 2-5: Predicted macrolides produced by KdesI mutants.

reductase can also stereospecifically reduce compound **23** to TDP-D-quinovose (**70**), also known as TDP-6-deoxy-D-glucose. The glycosyltransferase, DesVII, with its relaxed specificity toward sugar substrates, may catalyze the coupling of **70** to the aglycone **65**. The resulting product is expected to be further modified by a PikC-catalyzed hydroxylation to give new macrolides **71** and **72** containing D-quinovose. Thus, if a glycosylated product is successfully isolated, the structural analysis of its sugar moiety should provide significant insight into the biosynthetic pathway for the formation of D-desosamine.

Accordingly, the pDesI-d1 plasmid was constructed for the deletional disruption of *desI* (Figure 2-1). Two DNA fragments flanking *desI*, named "desI" and "sug-d", were amplified by PCR. These fragments, together with another DNA fragment, "neo", containing the neomycin resistance gene (also conferring resistance to kanamycin), were ligated into the *Hind*III-*Eco*RI sites of the vector pUC119. The 3.5 kb *Eco*RI-*Hind*III section of the resulting pDesI-d1 plasmid was subsequently cloned into the pKC1139 vector. The pKC1139 vector contains an apramycin resistance marker as well as a conjugal transfer gene, *oriT*, and is used for the transfer of DNA from *E. coli* to *Streptomyces* strains.[18] The resulting plasmid, pDesI-K, (Figure 2-1) was used to transform *E. coli* S17-1, which serves as the donor strain to introduce the pDesI-K construct *via* conjugal transfer into the wild-type *S. venezuelae*. As shown in Figure 2-6, the homologous recombination between pDesI-K and the *S. venezuelae* chromosome led to an alteration of the *S. venezuelae* chromosome. The mutants in which *neo* was

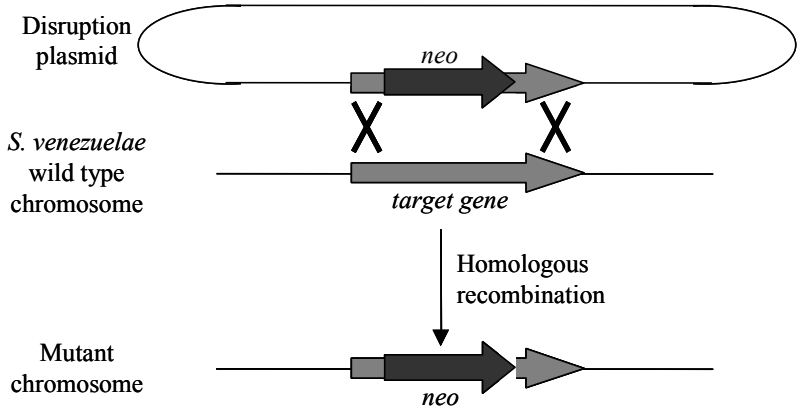


Figure 2-6: Replacement of the target gene with antibiotic resistance marker (*neo*) *via* homologous recombination.

inserted in the chromosomal *desI* gene were selected according to their kanamycin-resistant (Kan^R) and apramycin-sensitive (Apr^S) characteristics.

One colony, KdesI-80, was chosen for further study. This mutant was grown in the methymycin production medium (8 L),[13] and its metabolites were extracted with chloroform and analyzed by TLC. A preliminary TLC analysis revealed that no methymycin (**1**) or neomethymycin (**2**) were produced. However, a comparison of the extracts of the wild-type strain with extracts from KdesI-80 resulted in the appearance of several new spots that may correlate to novel macrolides (Figure 2-7, lane 1).

The crude extract (yellow oil) was subjected to flash chromatography on silica gel, and the isolated products were further purified by HPLC. Indeed, no methymycin or neomethymycin were detected. Instead, 10-deoxymethynolide (**65**) was found as the major product (approximately 600 mg). Significant quantities of methynolide (**74**) (approximately 40 mg) and neomethynolide (**75**)

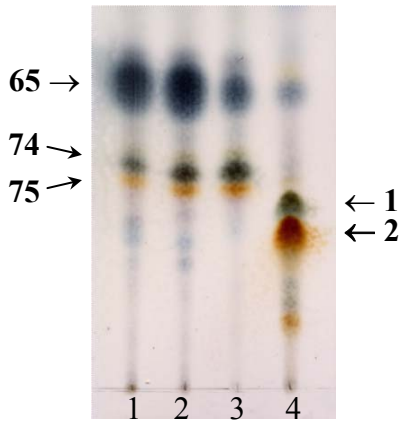
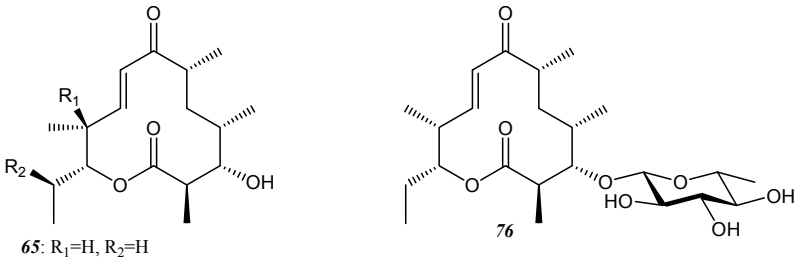


Figure 2-7: TLC analysis of macrolide production by *S.venezuelae* strains: KdesI-80 (lane 1), KdesII-101 (lane 2), KdesVIII-92 (lane 3), and wild type (lane 4). The major products are labeled: 10-deoxymethynolide (**65**), methynolide (**74**), neomethynolide (**75**), methymycin (**1**), and neomethymycin (**2**).

(approximately 2 mg) were also isolated. These compounds were identified by comparison of their NMR data with published spectra for **65**,[27] **74**,[28, 29] and **75**.[30] Most importantly, a new macrolide **76** containing D-quinovose (3.2 mg) was produced by this mutant. The structure of **76** was established by spectral analyses (^{1}H NMR, ^{13}C NMR, COSY, HMQC, and high resolution FAB-MS). Compounds **65** and **74–76** are shown in Figure 2-8.

The fact that the macrolide **76** containing D-quinovose is indeed produced by the *desI* mutant is significant. Firstly, the formation of quinovose as predicted further corroborates the presence of a pathway-independent reductase in *S. venezuelae* that reduces 3-keto sugars. Interestingly, this reductase is able to act on the 4,6-dideoxy sugar **20** (Section 1.5), as well as the 6-deoxy sugar **23**, suggesting that it is insensitive to the presence of a hydroxyl group at C-4. It is



65: R_1=H, R_2=H
methynolide, ***74***: R_1=OH, R_2=H
neomethynolide, ***75***: R_1=H, R_2=OH

Figure 2-8: Isolated macrolide products produced by KdesI-80 mutant.

also possible that multiple pathway-independent reductases exist in *S. venezuelae*. However, it is not clear whether the reduction occurs before or after the monosaccharide is appended to the aglycone (Figure 2-5).

Secondly, the retention of the 4-OH in quinovose as the result of the *desI* deletion provides strong evidence supporting the assigned role of *desI* as encoding a C-4 dehydrase. This finding also lends further credence to the proposed biosynthetic pathway in which C-4 deoxygenation precedes C-3 transamination.[9, 10] An alternative pathway,[6, 8] where the order of these two steps is reversed, is incompatible with the current observation.

Furthermore, the data again shows that the glycosyltransferase (DesVII) of this pathway can recognize alternative sugar substrates whose structures are considerably different from the original amino sugar substrate, desosamine. While the incorporation of quinovose is important, another noteworthy, albeit unexpected, result is the fact that the aglycone of the isolated macrolide (**76**) is 10-deoxymethynolide (**65**) instead of methynolide (**74**) or neomethynolide (**75**). It appears that PikC, which catalyzes the hydroxylation of 10-deoxymethynolide aglycone at its C-10 or C-12 position (Section 1.5), is sensitive to structural

variations in the appended sugar. It could be argued that the presence of the 4-OH group in the sugar moiety is somehow responsible for decreasing or fully preventing the hydroxylation of the macrolide.

2.3.2 Inactivation of *desII via* Partial Gene Deletion

On the basis of the assumption that DesII, a putative reductase, may assist DesI to effect a C-4 deoxygenation in a manner similar to the C-3 deoxygenation catalyzed by E_1/E_3, the disruption of the *desII* gene should result in the accumulation of a Schiff base adduct (Figure 1-17). However, since this adduct is expected to exist in an equilibrium with **23**, the pathway-independent C-3 keto reductase should stereospecifically reduce **23** to generate D-quinovose as is the case with the *desI* disruption. Therefore, it is likely that the macrolide **76**, produced by the KdesI-80 mutant, may also be produced by the *desII*-deleted mutant.

We used the same methodology as for the *desI*-deleted mutant in order to create a *desII*-deleted mutant. First, the pDesII-d1 plasmid was constructed, containing two fragments flanking *desII*, "desII-up" and "desII-down", as well as "neo", ligated into the *Eco*RI-*Hind*III sites of the vector pUC119. In this case, PCR was not successful for the "desII-down" fragment. Therefore, this fragment was obtained by digestion of the pL005 plasmid containing a part of the *des* gene cluster, specifically *desI*, *desII*, and *desIII* genes.[15] The 4.5 kb *Eco*RI-*Hind*III fragment of the plasmid pDesII-d1 was subsequently cloned into the pKC1139 vector giving pDesII-K. This latter plasmid was introduced into the wild-type *S. venezuelae via* conjugal transfer.

Using the same methodology as for the *desI*-deleted mutant, the KdesII-101 mutant was subsequently created, in which the *desII* gene was replaced by *neo*. The macrolide products (Figure 2-7, lane 2) were isolated from KdesII-101 (3 L of culture), and their structures were established by spectral analyses. These metabolites included previously identified aglycones **65** (the major product), **74**, and **75** (minor products) (Figure 2-8). However, no **76** was found in the fermentation broth. Instead, a YC-17 derivative **77** (2.4 mg) and a pikromycin derivative **78** (approximately 1 mg) were isolated. Both compounds carry an acetylated 4-aminosugar (Figure 2-9).

The production of macrolides **77** and **78** by the KdesII-101 mutant was completely unexpected. On the basis of the proposed biosynthetic pathway for TDP-D-desosamine, no route exists for the generation of such a C-4 amino sugar intermediate. The presence of a pathway-independent C-4 aminotransferase is not likely since the KdesI-80 mutant did not produce compounds **77** and **78**.

In light of the surprising outcome of the experiment, we decided to verify that the mutation introduced was correct. A Southern blot hybridization analysis was done using the "neo" or the 0.95 kb "desII-probe" fragment. The latter was amplified by PCR from the *desIII* region of *S. venezuelae*. As illustrated in Figure

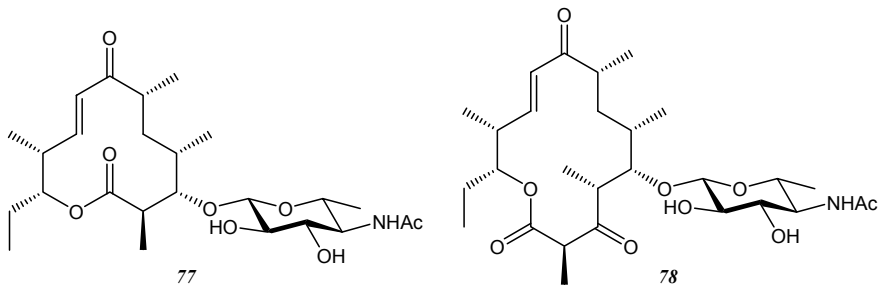


Figure 2-9: Isolated macrolide products produced by KdesII-101 mutant.

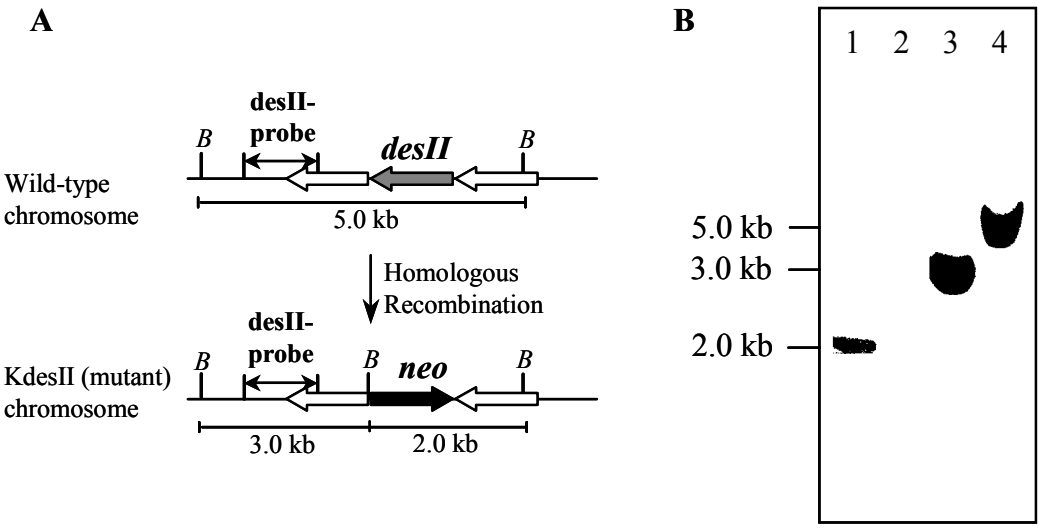


Figure 2-10: Disruption of the *desII* gene of *S. venezuelae*. (A) Generation of the *desII*-disrupted KdesII-101 mutant *via* a homologous recombination. The *Bam*HI restriction site is abbreviated as *B*. (B) Southern blot analysis of *Bam*HI digested genomic DNA of *S. venezuelae* wild-type and the KdesII-101 mutant. Lanes 1 (KdesII-101) and 2 (wild-type) were probed by the 1.4 kb "neo" DNA fragment; lanes 3 (KdesII-101) and 4 (wild-type) were probed by the 0.95 kb "desII-probe" fragment.

2-10, when the *Bam*HI-digested chromosomal DNA prepared from the mutant KdesII-101 was probed with the ^{32}P-labeled "neo", a 2.0 kb *Bam*HI band was detected, while no such band was detected in the *S. venezuelae* wild-type strain. With the "desII-probe", a 3.0 kb *Bam*HI band was detected for the mutant strain, while a 5.0 kb *Bam*HI band was revealed in the wild-type DNA. This experiment confirmed that the *desII* gene was indeed replaced by *neo* on the chromosome of the KdesII mutant.

As confirmed by Southern blot hybridization, the production of the macrolides **77** and **78** containing an acetylated 4-aminosugar must be the result of

disruption of the *desII* gene in the KdesII-101 mutant. A plausible explanation is that DesI may possess aminotransferase activity. However, whether this activity is an intrinsic property of DesI, or an activity exhibited by DesI in the absence of DesII, can only be established by further biochemical studies. Nonetheless, the observation of a C-4 amino sugar as a part of the macrolide metabolites produced by the *desII* mutant represents the first evidence that is inconsistent with the proposed biosynthetic pathway for TDP-D-desosamine (**22**) shown in Figure 1-17.

2.3.3 Inactivation of *desVIII via* Partial Gene Deletion

The assignment of DesVIII as the tautomerase catalyzing the conversion of TDP-4-keto-4,6-dideoxyhexose **18** to its 3-keto analog **23** (Figure 1-17) was a speculation based solely on chemical intuition. An inactivation of the *desVIII* gene, however, should shed light on this assignment, provided that the predicted sugar intermediate (4-keto-6-deoxy sugar, or D-quinovose) can be incorporated into the macrolides produced by the *desVIII*-deleted mutant.

The KdesVIII-92 mutant was subsequently created, in which the *desVIII* gene was replaced by *neo*, using the method described for the *desI* deletion. After fermentation, aglycones **65**, **74**, and **75** were isolated (Figure 2-7, lane 3). However, no glycosylated macrolides were found despite repeated attempts. A Southern blot hybridization analysis was used to confirm that *desVIII* is indeed replaced by *neo* in KdesVIII-92. The genomic DNA of the wild type *S. venezuelae* and the KdesVIII-92 mutant were isolated, digested with restriction endonucleases *Bam*HI and *Pst*I, and analyzed using Southern blot hybridization. As expected, no hybridization of the neomycin resistance gene with the wild type

DNA was observed. Meanwhile, a prominent band of 1.4 kb corresponding to the approximate size of the *neo* gene was detected on the autoradiogram of the mutant DNA. In a separate experiment, the 1.0 kb *Nco*I-*Xho*I fragment of the desosamine gene cluster outside the *desVIII* gene was used as a probe. The bands corresponding to 4.7 kb and 5.5 kb hybridized fragments were expected for the *Bam*HI-*Pst*I fragment derived from the mutant and the wild type DNA, respectively. Indeed, these bands were detected on the autoradiogram. Therefore, the replacement of *desVIII* with *neo* was successful.

One of the possible explanations for the absence of glycosylated compounds is that the disruption of *desVIII* has a polar effect on the downstream *desVII*. If this is the case, the *desVII* gene will not be transcribed. Consequently, no glycosyltransferase will be synthesized resulting in an inability to glycosylate any macrolides.

In order to test this hypothesis, an extra copy of the *desVII* gene was added into the KdesVIII-92 mutant. The 1.3 kb *desVII* gene was amplified by PCR using synthetic oligonucleotide primers and the pLZ4 cosmid as the template. The amplified gene was cloned into the *Eco*RI-*Xba*I restriction sites of the expression vector pDHS617, which contains an apramycin resistance marker (Apr^R). The plasmid pDHS617 is derived from pOJ446.[18] It contains the promoter sequence from the methymycin/neomethymycin cluster[14] to drive the expression of genes in *S. venezuelae*. The resulting plasmid, pDesVII (Figure 2-4), was introduced by conjugal transfer using *E. coli* S17-1[18] into a previously constructed *S. venezuelae* mutant KdesVIII-92. The colonies of *S. venezuelae*

KdesVIII containing pDesVII were identified on the basis of their resistance to both apramycin and kanamycin. One of the engineered strains, *S. venezuelae* desVII/KdesVIII, was chosen for further study and its fermentation products were isolated as described above. Again, only aglycones were isolated, and no macrolides containing a sugar moiety were observed. Judging by TLC analysis, the phenotype of the desVII/KdesVIII mutant was identical to that of KdesVIII-92. Thus, no polar effect exists on the genes located downstream of *desVIII*. In a separate experiment, a copy of the *desVIII* gene was introduced into KdesVIII-92 using the same experimental procedure. The phenotype (based on TLC analysis) of the resulting strain desVIII/KdesVIII closely resembles that of the wild type *S. venezuelae*, suggesting again that the expression of the downstream genes is not affected by the deletion of *desVIII*. Therefore, the absence of glycosylated products is not caused by the polar effect.

The fact that only aglycones were produced by the *S. venezuelae* KdesVIII and desVII/KdesVIII mutants strongly suggests that *desVIII* is involved in deoxy sugar biosynthesis and/or the attachment of deoxy sugars to the aglycones. Whether it catalyzes a specific reaction in the pathway is yet unclear.

2.3.4 Implications on the Revised Biosynthetic Pathway for D-Desosamine

The isolation and characterization of macrolide **76** produced by the *desI*-deleted mutant have provided direct evidence confirming the involvement of *desI* in the C-4 deoxygenation step and the initial reaction sequence of the desosamine pathway. The disruption of *desII*, resulting in the isolation of the macrolides **77** and **78** containing an acetylated 4-aminosugar suggested that DesI may possess

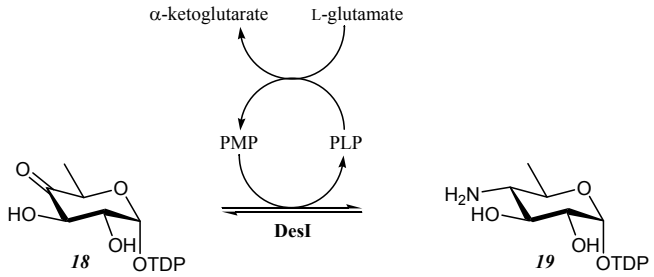


Figure 2-11: C-4 transamination reaction catalyzed by DesI in the presence of L-glutamate and PLP.

C-4 aminotransferase activity. Indeed, recent biochemical studies conducted in our group confirmed this hypothesis.[15, 31] As shown in Figure 2-11, when compound **18** was incubated with the recombinant DesI in the presence of an amino donor (L-glutamate) and PLP, the 4-aminosugar **19** was isolated. This result firmly established, for the first time, the function of DesI as the PLP-dependent aminotransferase catalyzing the conversion of **18** to **19** in the biosynthesis of TDP-D-desosamine. Compounds **77** and **78** are likely derived from the coupling of **19** and the corresponding aglycone, followed by *N*-acetylation. It is also conceivable that *N*-acetylation of **19** occurs prior to its coupling to the aglycones. Regardless of the sequence of events, the production of **77** and **78** indicates that **19** must be accumulated in the *desII*-deleted mutant of *S. venezuelae*. It can also be concluded that DesII is most likely the enzyme whose action occurs immediately after that of DesI in the desosamine biosynthetic pathway.

The fact that DesI catalyzes the conversion of **18** to **19** has important implications on the biosynthesis of desosamine. Based on this new evidence, the

tautomerization of **18** to **23** is no longer a necessary step in the pathway (Figure 1-17). This conclusion is also supported by the *desVIII* disruption study. Since **23** is not a part of the pathway, the originally proposed mechanism of the C-4 deoxygenation is no longer possible. Therefore, a new mechanism, which involves a 4-amino sugar intermediate (**19**), must be considered for the C–O bond cleavage at C-4.

The genetic studies described herein, combined with the biochemical studies on DesI, have allowed us to propose a revised biosynthetic pathway for TDP-D-desosamine (Figure 2-12). In this pathway, TDP-4-keto-6-deoxyglucose (**18**), generated from glucose-1-phosphate in two consecutive steps catalyzed by DesIII and DesIV, is converted to TDP-3-keto-4,6-dideoxyglucose (**20**) *via* a 4-amino sugar intermediate (**19**) catalyzed by DesI and DesII. The subsequent amination of **20** by DesV leads to TDP-3-amino-3,4,6-trideoxyglucose (**21**). Finally, the DesVI-catalyzed *N,N*-dimethylation of **21** completes the biosynthesis of TDP-D-desosamine (**22**). A full understanding of the mechanistic aspects of

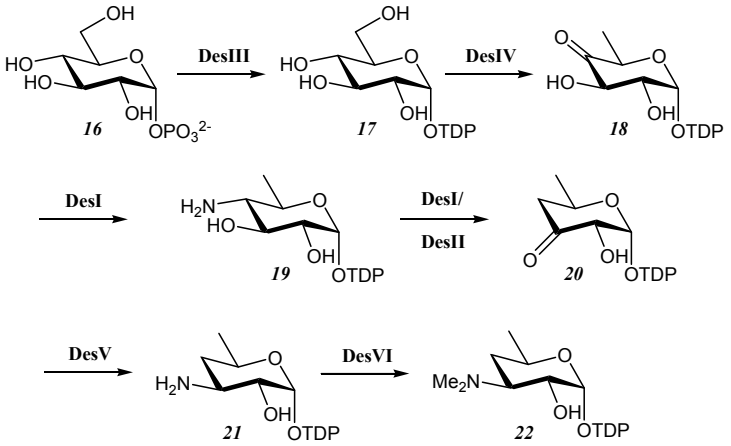


Figure 2-12: The biosynthetic pathway for TDP-D-desosamine (**22**) by *S. venezueale*.

the desosamine formation, in particular the transformation from **19** to **20**, must await further biochemical investigations.

2.3.5 Implications on the Genetic Engineering of Methymycin/Neomethymycin Analogs

Manipulations of the desosamine biosynthetic genes of *S. venezuelae* have led to the construction of several macrolides that carry two different deoxy sugars, D-quinovose and 4,6-dideoxy-4-acetamido-D-glucose. The work presented here demonstrates the feasibility of combining pathway-dependent genetic manipulations and pathway-independent enzymatic reactions as a means to engineer sugars of designed structures. The fact that pathway-independent enzymes could be used in concert with the natural biosynthetic machinery to generate further structural diversity is especially encouraging. Most importantly, the methymycin/neomethymycin glycosyltransferase, DesVII, has been shown to be promiscuous, tolerating significant structural variations of its sugar substrates.

The deletion of *desI* in *S. venezuelae* led to the accumulation of the 4-keto intermediate **18**, a common precursor for bacterial deoxy sugars. Thus, the *S. venezuelae* KdesI mutant may serve as a useful host for the construction of "tailored" deoxy sugar biosynthetic machineries which use **18** as the initial precursor. Several proof of principle experiments have been carried out in our group and others. For example, Zhao *et al.* have expressed the *calH* gene, involved in the calicheamicin biosynthesis by *Micromonospora echinospora* spp. *Calichensis*, in the *S. venezuelae* KdesI mutant.[19] The resulting hybrid strain produced the same glycosylated products, **77** and **78** (Figure 2-9), as those produced by the *S. venezueale* KdesII mutant. This result confirms that the

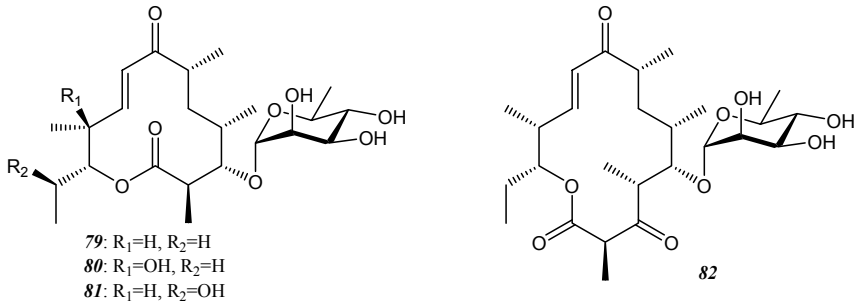


Figure 2-13: Macrolides produced by *S. venezuelae* KdesI carrying both *strM* and *strL* genes.

function of *calH* is the same as that of *desI*, namely a C-4 aminotransferase.[19] Another example is reported by Yamase *et al.*, in which two genes of the L-dihydrostreptose biosynthetic pathway in the streptomycin producer *Streptomyces griseus*, *strM* and *strL*, were introduced into the *S. venezuelae* KdesI mutant.[32] The *strM* and *strL* genes have been proposed to encode the TDP-4-keto-6-deoxyglucose-3,5-epimerase and the dihydrostreptose synthase, respectively. The resulting strain produced four new macrolides, **79**–**82**, all of which contain an L-rhamnose residue (Figure 2-13). This study demonstrated the capability of DesVII to recognize and process sugar substrates possessing an L-hexose configuration.

It is hoped that the knowledge and experience gained from the ongoing research of unusual sugar biosynthesis will facilitate the development of novel drugs against bacterial infections based on a combinatorial biosynthetic approach.

2.3.6 Replacement of *desVIII* with Its Homologues from Other Pathways

The deduced product of *desVIII* displays a significant sequence similarity to the gene products of *eryCII* (31% identity) in the erythromycin gene cluster of

S. erythraea,[7, 8] *tylM3* (34% identity) in the tylosin gene cluster of *S. fradiae*,[33], and *dnrQ* (35% identity, also known as *dnmQ*) in the aromatic polyketide daunorubicin (**84**, Figure 2-14 B) gene cluster of *S. peucetius*,[34, 35] among several others. These enzymes have been proposed to be involved in the biosynthesis of the deoxyamino sugars attached to their respective aglycones: EryCII in the desosamine pathway, TylM3 in the mycaminose pathway, and DnrQ in the daunosamine pathway. While their functions remain uncertain, EryCII has been suggested to serve as a tautomerase catalyzing the conversion of the 4-keto sugar **18** to the corresponding 3-ketosugar **23**.[7] However, it should be noted that the biosynthetic pathway to daunosamine does not necessarily require a tautomerization step.[35] In fact, it has been shown that the formation and the attachment of daunosamine was significantly diminished, but not abolished, when *dnrQ* gene was inactivated.[36] Since all four gene display significant sequence similarity, the fact that a tautomerization step may not be required in the biosynthesis of TDP-L-daunosamine raises questions as to the actual roles of the gene products of *eryCII*, *tylM3*, *dnrQ*, and *desVIII*.

The gene disruption results described above led to the proposal of a new pathway to TDP-D-desosamine (**22**) (Figure 2-12) in which the DesI/DesII couple catalyzes the transformation of **18** to **20**. This revised biosynthetic pathway does not provide a role for DesVIII. However, the fact that *desVIII* is located within the desosamine gene cluster (Figure 1-15) implicates a role of DesVIII in either the biosynthesis of desosamine or its attachment to the macrolide aglycone. The inability of the *S. venezuelae* KdesVIII mutant to produce glycosylated products

further confirmed the importance of *desVIII* for the complete biosynthesis of the macrolides.

Encouraged by the successful expression of heterologous genes in the *S. venezuelae* KdesI mutant, we decided to probe whether *desVIII* and its homologues, *eryCII*, *tylM3*, and *dnrQ*, have similar functions. Our approach is to explore the complementarity of these genes to *desVIII* in the *S. venezuelae* KdesVIII mutant.

Plasmids pEryCII, pTylM3, and pDnrQ were created, in which the genes *eryCII*, *tylM3* and *dnrQ*, respectively, were cloned into the *Streptomyces* expression vector pDHS617. The plasmids were introduced into the *S. venezuelae* KdesVIII-92 mutant *via* conjugal transfer. The secondary metabolites produced by the resulting mutant strains, eryCII/KdesVIII, tylM3/KdesVIII, and dnrQ/KdesVIII, were then subjected to TLC analysis.

Two of the mutant strains, eryCII/KdesVIII and tylM3/KdesVIII, produced only aglycone products **65**, **74**, and **75**, indicating that their phenotypes were identical to that of the KdesVIII-92 strain. No methymycin, neomethymycin, or other glycosylated analogs were detected in these strains. The inability of the eryCII/KdesVIII mutant to produce glycosylated compounds is a surprise, since *eryCII* and *desVIII* are found in the biosynthetic gene clusters of the same sugar product, TDP-D-desosamine (**22**) in *S. erythraea* and *S. venezuelae*, respectively. Given the substantial degree of sequence similarity between these two genes, they are expected to have the same catalytic function(s) and to work on the same substrate if both are directly involved in the formation of

desosamine. However, the above negative complementation results suggested that they may catalyze the same reaction, but have different substrate preferences. A possible scenario is that DesVIII and EryCII are involved in the attachment of desosamine to the respective aglycone, not the construction of desosamine itself. The differences in the structures of 10-deoxymethynolide (**65**) and mycarosylated erythronolide B (**43**) may contribute to the lack of complementarity between EryCII and DesVIII. A similar argument can also be applied to TylM3.

Unlike the cases of eryCII/KdesVIII and tylM3/KdesVIII, a TLC comparison of the crude extract of the dnrQ/KdesVIII strain with that of the wild type *S. venezuelae* showed that dnrQ/KdesVIII is able to produce methymycin and neomethymycin, albeit in lower quantities than the wild type strain. The crude extract of dnrQ/KdesVIII culture (3 L) was subjected to separation on a silica gel column followed by HPLC purification. As before, the major product was 10-deoxymethynolide (**65**, approximately 40 mg), but hydroxylated aglycones **74** and **75** were also present (approximately 2–5 mg each). In addition, we isolated approximately 3 mg each of pure methymycin (**1**) and neomethymycin (**2**), as well as approximately 4 mg of crude pikromycin (**4**). Interestingly, we also isolated approximately 7 mg of a new product, compound **83** (Figure 2-14 A). Compound **83** contains the same sugar moiety as **1** and **2**, D-desosamine, but has two positions of the aglycone, C-10 and C-12, hydroxylated instead of one.

While the production of **1** and **2** by the dnrQ/KdesVIII mutant is only 1/10 as efficient as the wild type, the fact that the wild type products **1**, **2**, and **4** were

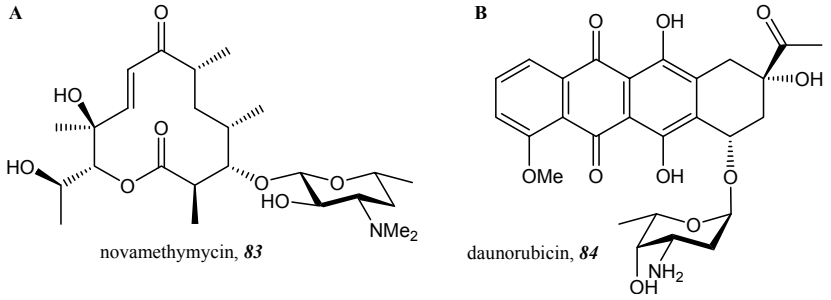


Figure 2-14: (A) New macrolide compound produced by the *S. venezuelae* dnrQ/KdesVIII strain, novamethymycin (**83**). (B) The structure of daunorubicin (**84**) produced by *S. peucetius*. The homologue of *desVIII* in *S. peucetius*, *dnrQ*, is involved in the biosynthesis of **84**.

produced indicates that *dnrQ* can at least partially replace *desVIII*. The lower yield of **1** and **2** may be attributed to the less efficient expression of the plasmid gene as compared to the expression of the genes located on the chromosomal DNA. It may also be due to the intrinsic differences in the biochemical properties of these two proteins. Significantly, compound **83** was obtained in quantities comparable to or even larger than those of **1** and **2**.

Shortly after we completed the characterization of the metabolites produced by the *S. venezuelae* dnrQ/KdesVIII strain, a report was published describing the isolation and characterization of **83** as a minor component produced by the wild type *S. venezuelae*.[37] It was shown that **1** was converted to **83** by PikC *in vitro*, providing insight into how **83** is formed in the wild type *S. venezuelae*.[37] Thus, our discovery of **83** as one of the major glycosylated products produced by *S. venezuelae* dnrQ/KdesVIII strain, along with **1** and **2**, reflects only the change in the ratios of the wild type metabolites, not their compositions.

Although *desVIII* and *dnrQ* were originally proposed to be involved in deoxy sugar biosynthesis or attachment, the results of this experiment clearly demonstrated that the replacement of *desVIII* with *dnrQ* can influence the extent of hydroxylation of the aglycone. It is unlikely that these proteins directly participate in the hydroxylation. Instead, *desVIII* and its homologues may have a regulatory function and their expression affects the hydroxylation as well as the glycosylation catalyzed by the corresponding P450 hydroxylase and glycosyl-transferase in the respective pathways. Clearly, the heterologous expression and the analysis of the catalytic function of DesVIII *in vitro* would be helpful. The detailed biochemical characterization of DesVIII will be discussed in Chapter 4.

2.4 CONCLUSIONS

The results of the *desI* and *desII* gene disruptions, combined with the biochemical studies of DesI, led to the establishment of the function of DesI as a PLP-dependent aminotransferase. It catalyzes the conversion of the 4-keto sugar substrate **18** to an amino sugar intermediate **19** during the biosynthesis of TDP-D-desosamine (**22**). A revised pathway, which involves a 4-aminosugar intermediate **19**, was proposed on the basis of these studies (Figure 2-12). This pathway may be a general route for the biosyntheses of other 4,6-dideoxyhexoses. The disruption of *desVIII* and its replacement with homologues from different pathways showed that although this gene is involved in the deoxysugar biosynthesis or/and attachment, DesVIII unlikely catalyzes a specific chemical transformation in the pathway. Rather, DesVIII may serve a regulatory function.

Moreover, methymycin analogs carrying altered deoxy sugar moieties were generated *via* genetic engineering. These results demonstrated the feasibility of combining pathway-dependent transformations and pathway-independent enzymatic reactions as a means to tailor-make sugars of the designed structures. In addition, the relaxed specificity of the glycosyltransferase DesVII was also demonstrated.

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Chapter 3: Secondary Self-Protection Mechanism in *S. venezuelae*

3.1 INTRODUCTION

Three genes involved in self-resistance have been identified in the methymycin/pikromycin biosynthetic gene cluster of *S. venezuelae* (Chapter 1).[1] Two of them, *pikR1* and *pikR2*, are MLS_B type resistance genes. They encode methyltransferases responsible for the methylation of a specific adenosine residue near the macrolide binding site in the rRNA. The third one, *desR*, encodes a *β*-glycosidase whose function was not apparent when this gene was first identified.[2] Because of the presence of this gene in the cluster, it was speculated that the macrolide antibiotics produced by *S. venezuelae* may be modified by a specific macrolide glycosyltransferase (MGT) to incorporate a glucose moiety in the final products. Such a modification of glycosylation may render the antibiotic in the cell inactive. The glucosylated macrolides may then be excreted together with the *β*-glycosidase DesR from the cell, where DesR catalyzes the hydrolysis of the

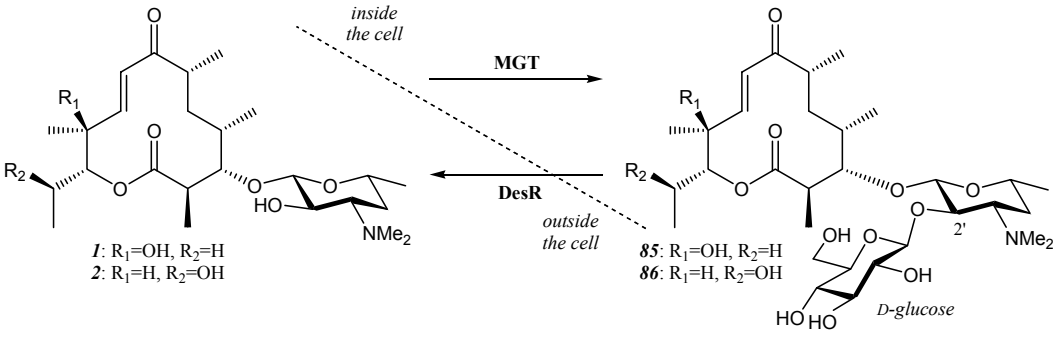

Figure 3-1: The mechanism of self-resistance in *S. venezuelae* through glucosylation of **1** and **2** by macrolide glycosyltransferase (MGT). DesR reactivates glucosylated products **85** and **86** outside the cell.

second glycosidic linkage, reverting the inactivated antibiotic to its active form (Figure 3-1).

The function of DesR as a β-glycosidase was initially verified by gene disruption experiments[2] and was later confirmed by *in vitro* assay of the purified DesR.[3] The isolation of **85** and **86** (Figure 3-1) is a strong indication for the presence of an MGT to catalyze the glucosylation of **1** and **2**. However, no glycosyltransferase genes other than *desVII* were found in the vicinity of the methymycin/pikromycin gene cluster of *S. venezuelae*. The *desR*-deleted *S. venezuelae* mutant still produces **1** and **2** as the major metabolites, indicating that the efficiency of the responsible MGT may be low. From this observation we were also able to suggest that perhaps the methylation of the rRNA by PikR1 and PikR2, is the primary resistance mechanism in *S. venezuelae*.[2] Whether the methylation of rRNA is indeed the primary resistance mechanism, or whether it only comes into play when the glucosylation-deglucosylation routine is hampered, remains to be elucidated.

The genetic and biochemical study of the resistance determinants in *S. venezuelae* is described in this chapter. The inviability of the *S. venezuelae* triple mutant lacking *pikR1*, *pikR2*, and *desR* suggested that the methylation of rRNA is the primary self-resistance mechanism. We also carried out an initial biochemical study of the MGT OleD from the oleandomycin producer *S. antibioticus*. Most significantly, we screened the genome of *S. venezuelae* for *oleD* homologue, and identified an MGT gene. The MGT of *S. venezuelae*,

DesG, was expressed in *E. coli* and its expected catalytic function was confirmed in a preliminary *in vitro* assay.

3.2 EXPERIMENTAL PROCEDURES

3.2.1 General

Bacterial Strains. *E. coli* DH5α from Invitrogen (Carlsbad, CA) was used throughout the study as the regular cloning host. *E. coli* S17-1[4] was the donor strain for the conjugal transfer to *S. venezuelae*. *E. coli* BL21(DE3) from Novagen Inc. (Madison, WI) was used as the host for the overexpression of *oleD* and *desR* gene. *E. coli* LE392 was used as the host for the construction of a cosmid library of the *S. venezuelae* genomic DNA. The strains *S. venezuelae* ATCC 15439 and *S. antibioticus* ATCC 11891 were obtained from American Type Culture Collection (ATCC, Rockville, MD) as freeze-dried pellets and were revived according to the instructions provided by ATCC. The strain *S. venezuelae* KdesR, in which the wild type *desR* gene is replaced with the thiostrepton resistance gene (through the homologous recombination between the wild type *S. venezuelae* and the disruption construct pBL1005) was previously prepared by a former group member Dr. Lishan Zhao.[2, 5]

Plasmids, Vectors and DNA Manipulations. The pME43 cosmid (the derivative of the pNJ1 vector)[6] containing *pikR1* and *pikR2* genes, as well as fragments of the polyketide synthase cluster[5] was used as the template for polymerase chain reaction (PCR) and as the source of the appropriate DNA fragments for the *pikR1*/*pikR2* disruption experiments. The pKC1139 plasmid, used for the conjugal transfer of DNA to *S. venezuelae*, and the cosmid vector

pNJ1 were gifts from Dr. Leonard Katz of Abbott Laboratories.[7] The pDHS617 vector, used for the complementation of the *S. venezuelae* KdesR strain with the *oleD* gene, was a gift from Professor David Sherman of the University of Michigan.[8] The pFD666 cosmid was used as the source of the *neo* fragment.[9, 10] The expression vectors pET24b(+) and pET28b(+) were obtained from Novagen. The general methods and protocols for recombinant DNA manipulations followed the description by Sambrook *et al.*,[11] and those dealing with *Streptomyces* strains followed the description by Hopwood *et al.*[12] and Kieser *et al.*[13]

Biochemicals. The enzymes used in the cloning experiments were obtained from Invitrogen or Promega (Madison, WI). The ^{32}P labeled nucleotides and the Multiprime DNA Labeling System used for the DNA probe labeling during the Southern blot hybridization analysis were purchased from Amersham Biosciences (Piscataway, NJ). The antibiotics and biochemicals used in this study were the products of Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA). The growth media components were obtained from BD Diagnostics System (Sparks, MD). The purification of DNA after digestion reactions and from an agarose gel was achieved using the Bio101 Geneclean II® kit purchased from Fisher Scientific.

The Ni-NTA resin for the affinity purification of His-tagged proteins was obtained from Qiagen (Valencia, CA). Glycosyltransferase Affinity Gel-UDP was purchased from EMD Biosciences Inc. (San Diego, CA). The DEAE Sepharose CL-6B resin for ion exchange and Sephacryl S200 HR resin for size exclusion protein chromatography were obtained from Amersham Biosciences

(Piscataway, NJ). All protein purification steps were carried out at 4 ^{o}C except for FPLC chromatography (Amersham Biosciences) that was performed at room temperature. FPLC was equipped with the ion exchange MonoQ HR 10/10 or the size exclusion Superdex 200 HR 10/30 column.

Instrumentation. The pH values were obtained using the Corning pH meter 240 purchased from Fisher Scientific. The mini-sub cell GT from BioRad (Hercules, CA), powered by either a FB600 or a FB135 power supply from Fisher Scientific, was used for the agarose gel electrophoresis. All centrifugation procedures were performed using an Avanti J-25 unit from Beckman (Arlington Heights, IL). Microcentrifugations were done with an Eppendorf 5415C from Brinkmann Instruments, Inc (Westbury, NY). The gel documentation was performed with a Polaroid MP-4 Land Camera (Cambridge, MA) loaded with a Polaroid Type 667 coatless black and white instant film and illuminated with a Foto UV 310 transilluminator from Fotodyne Inc (New Berlin, WI). The polymerase chain reactions were conducted using a Perkin Elmer Cetus DNA Thermal Cycler (Norwalk, CT). The ultraviolet–visible spectra were recorded with a Beckman DU-650 spectrophotometer. The HPLC separations were achieved on a Beckman 366 instrument (Beckman Instruments, Fullerton, CA) equipped with Econosil C_{18} columns from Alltech (Deerfield, IL). The NMR spectra were acquired on a Varian Unity 300 or 500 spectrometer, and the chemical shifts (δ in ppm) are given relative to those for Me_4Si (for ^{1}H and ^{13}C) with the coupling constants reported in hertz (Hz). The flash chromatography was performed on the Lagand Chemical silica gel (230–400 mesh) by the elution

with the specified solvents. The analytical thin-layer chromatography (TLC) was carried out on the Polygram Sil G/UV_{254} plates (0.25 mm) (Macherey-Nagel Inc., Easton, PA).

Preparation of Competent Cells and the PCR Amplification of DNA. Cells were made competent by the RbCl method,[11] following the procedure described in Chapter 2. The general procedure for the PCR amplification of DNA fragments was the same as these described in Chapter 2.

SDS-PAGE. The protein purity and an estimated molecular mass were obtained by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) at 25 mA per gel, using a discontinuous buffer system.[14] The separating gel and the stacking gel were 12% and 4% polyacrylamide, respectively. Prior to electrophoresis, the protein samples were heated for 5 min at 100 ^{o}C (or 10 min at 37 ^{o}C for the samples containing high imidazole concentrations) in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.0025% bromophenol blue. Electrophoresis of the denatured samples was run in 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS (pH 8.3) using the Mini-PROTEAN II vertical system from BioRad. Gels were stained with Coomassie blue (2.5 g/L of Coomassie Brilliant Blue G-250 in acetic acid : water : methanol, 1:4:5 by volume) and de-stained in ethanol : acetic acid : water (4:5:41 by volume).[15] The protein concentrations were determined according to Bradford,[16] using bovine serum albumin (BSA) as the standard and the dye reagent from BioRad.

3.2.2 Construction of *S. venezuelae* KdesR Strain Complemented with *oleD*

Construction of Plasmid pOleD-1. The pOleD-1 plasmid was constructed for the expression of the *oleD* gene in the *S. venezuelae* KdesR mutant. The 1.3 kb DNA fragment containing *oleD* was amplified by PCR using primers 5'-GCCGGAATTCGCAACGTCTCGTCTACC-3' (forward) and 5'-CGCG-TCTAGATCACAAAGCGGATCTC-3' (reverse) with the genomic DNA of *S. antibioticus* digested with *Eco*RI as the template. The PCR product was digested with *Eco*RI and *Xba*I (the restriction sites are underlined in the sequence of the primers) and cloned into the *Eco*RI/*Xba*I sites of the vector pDHS617,[8] containing the apramycin resistance marker, to give the pOleD-1 plasmid. This plasmid was used to transform *E. coli* S17-1 and then introduced into the KdesR-11 mutant.

Complementation of S. venezuelae KdesR Strain with oleD. The pOleD-1 plasmid was introduced into the *S. venezuelae* KdesR-11 strain *via* conjugal transfer as described in Chapter 2. Selected colonies of the *S. venezuelae* oleD/KdesR mutant were propagated on SPA plates containing apramycin (100 μg/mL) and thiostrepton (50 μg/mL), and spore suspensions were prepared for storage.

Analysis of Metabolites Produced by S. venezuelae oleD/KdesR. For the analysis of the metabolite production of the recombinant *S. venezuelae* oleD/KdesR, this strain was grown under the 12-membered ring production conditions. The metabolites were isolated from a small-scale culture and analyzed by TLC as described in Chapter 2. The standards of **85** and **86** for TLC

comparison were obtained by Dr. Lishan Zhao from the fermentation broth of *S. venezuelae* KdesR-11.[2]

3.2.3 Construction of the Triple KdesR/KpikR1,R2 Mutant of *S. venezuelae*

Construction of the Disruption Plasmid pPikR-K. The pPikR-K plasmid was constructed and used to replace the *pikR1* and *pikR2* genes with the neomycin resistance gene (which also confers the resistance to kanamycin) in the genome of *S. venezuelae* KdesR strain to eliminate the PikR1 and PikR2 activities. The genes *pikR1* and *pikR2* are clustered and located upstream of the PKS cluster.[1] The two sequence fragments flanking *pikR1* and *pikR2* genes were amplified by PCR using primers: 5'-CGGACACGAATTCCTCGGAC-3' and 5'-GCGC-TCTAGAATACGGTGACCCTCTCC-3' for the "*pikR1* end" (1.1 kb long) and 5'-CGCGAAGCTTTGCAGCATGTTCCTGTCC-3' and 5'-GGTACTGCAG-GAAGTCGTGG-3' for the "*pikR2* end" (1.3 kb long). These primers contain the recognition sites (underlined) for the restriction enzymes *Eco*RI, *Xba*I, *Hind*III, and *Pst*I, respectively. The pME43 cosmid,[5] which contains the *pikR1* and *pikR2* genes as well as a part of the polyketide synthase gene cluster, was used as the template for the PCR amplification. To amplify the fragment "neo" containing the neomycin resistance gene (1.4 kb long) from the pFD666 plasmid,[10] the following primers were used: 5'-CGCGTCTAGATACCTACAGCGTGAGC-3' and 5'-CGCGCTGCAGCCACGAATTAGCC-3' (the restriction sites for *Xba*I and *Pst*I, respectively, are underlined).

The PCR products were digested with the corresponding restriction enzymes and ligated into the *Eco*RI and *Hind*III sites of pUC119 to give the

pPikR-d plasmid. The product of the four-way ligation, pPikR-d, was then digested with the *Eco*RI and *Hind*III enzymes and the resulting 3.74 kb fragment was cloned into pKC1139 affording the disruption plasmid pPikR-K, which conferred the resistance to both apramycin and kanamycin. Following the same procedure as for the construction of the KdesI, KdesII, and KdesVIII mutants (Chapter 2), this plasmid was used in the homologous recombination with the chromosomal DNA of the *S. venezuelae* KdesR-11 mutant to create the triple disruption mutant of *S. venezuelae*, KdesR/KpikR1, R2.

Screening of the Triple KdesR/KpikR1,R2 Mutant of S. venezuelae. The conjugal transfer of pPikR-K into the *S. venezuelae* KdesR-11 and screening for double-crossover mutants were carried out as described in Chapter 2 with the following exceptions. First, the *S. venezuelae* KdesR-11 recipient strain was used in place of the wild-type strain. Second, thiostrepton (20 μg/mL) was used for the propagation of the recipient and the resulting mutant cultures. The conjugation plates were flooded with 1 mL of an aqueous solution of nalidixic acid (500 μg/mL), apramycin (500 μg/mL), kanamycin (500 μg/mL), and thiostrepton (500 μg/mL) per plate. Throughout the screening, individual colonies exhibiting thiostrepton resistant ($Thio^R$), kanamycin resistant (Kan^R), and apramycin sensitive (Apr^S) characteristics were identified as double-crossover mutants KdesR/KpikR1, R2.

3.2.4 Expression of *oleD* and Purification of the Recombinant Protein

Construction of the Expression Plasmid pOleD-2. The *oleD* gene was expressed as a recombinant protein with a *C*-terminal His_6 tag. The *oleD* gene

was amplified by PCR using the following primers: forward primer 5'-GCCG-CATATGACCACCCAGACCACAC-3', containing an *Nde*I restriction site (underlined), and reverse primer 5'-GCGCAAGCTTCAAAGCGGATCTCTGC-3', containing a *Hind*III restriction site (underlined). The genomic DNA of *S. antibioticus* digested with *Eco*RI was used as the template. The PCR-amplified DNA fragment was cloned into the *Nde*I-*Hind*III sites of the vector pET24b(+) to give pOleD-2. This plasmid was used to transform *E. coli* BL21(DE3).

Cell Growth. An overnight culture of *E. coli* BL21(DE3)/pOleD-2 was used to inoculate five 1 L cultures (0.2% v/v) of the LB medium containing kanamycin (35 μg/mL). The cultures were incubated at 37 ^{o}C until the OD_{600} reached 0.5. The temperature of the incubation was lowered to 25 ^{o}C and the cultures were induced with 0.1 mM IPTG, and incubation at 25 ^{o}C continued for 16 h. The cells were harvested by centrifugation (5,000 g, 15 min).

Crude Extract Preparation. Cell pellet was re-suspended in 85 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 15% glycerol, pH 8.0) containing 2 mg/mL of lysozyme and incubated on ice for 30 min. The cells were disrupted by sonication in five 1-min bursts with a 1-min cooling period between each blast. Cell debris was removed by centrifugation (35,000 g, 30 min), and the supernatant was used in the next step.

Affinity Purification Using Ni-NTA Resin. The supernatant was mixed by a slow agitation with 3 mL of the packed Ni-NTA agarose resin (Qiagen, Valencia, CA) for 1 h at 4 ^{o}C. The slurry was poured into a capped column and washed with 120 mL of a lysis buffer. It was followed by washing with 60 mL of

the lysis buffer that contained 15 mM imidazole and 30 mL of the lysis buffer that contained 20 mM imidazole. The desired protein eluted in the flow-through fractions. It was later determined that due to the mutation in the pET24b(+) vector sequence, a stop codon was introduced upstream of the sequence coding for the His_6 tag. Consequently, the recombinant OleD protein did not have a His_6 tag. Therefore, it was purified using DEAE anion exchange chromatography.

Anion Exchange Chromatography Using DEAE Resin. The sample selected after the Ni-NTA purification was loaded onto a DEAE Sepharose CL-6B column (70 mL bed volume) and washed with 100 mL of a 50 mM potassium phosphate (pH 7.5) containing 15% glycerol. The bound proteins were then eluted using a linear gradient between equal volumes (1 L) of the loading buffer containing 0.2 M KCl and 0.5 M KCl. The fractions containing proteins (as

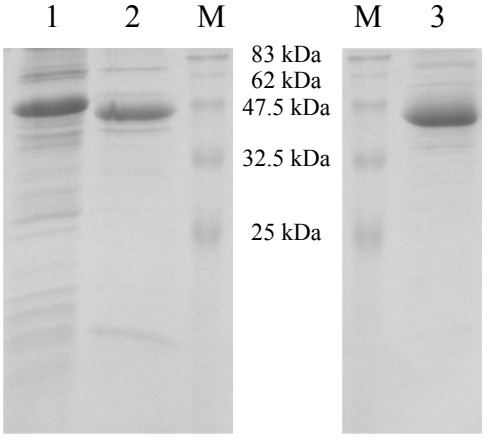

Figure 3-2: SDS-PAGE of proteins described in this chapter. Lane 1 is DesG-N-His (major band of approximately 46 kDa). Lane 2 is DesG-C-His (major band of approximately 45.5 KDa). Lane 3 is purified OleD (major band of approximately 48 kDa). Lane M is the prestained molecular weight marker and bands are as follows: *E. coli* MBP-paramyosin, 83 kDa; bovine glutamic dehydrogenase, 62 kDa; rabbit aldolase, 47.5 kDa; rabbit triosephosphate isomerase, 32.5 kDa; bovine β-lactoglobulin A, 25 kDa.

judged by the UV A_{280} readings) were further analyzed by SDS-PAGE. The desired fractions containing the major protein of 48 kDa were pooled and dialyzed against two 1 L changes of the 50 mM potassium phosphate buffer (pH 7.5) containing 15% glycerol. The purified OleD enzyme (Figure 3-2, lane 3) was aliquotted and stored at –80 ^{o}C.

3.2.5 OleD Activity Assay

In order to assay the glycosyltransferase activity of OleD, a TLC assay was used. The assay mixture consisted of 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.5 mM methymycin (**1**), 2 mM UDP-glucose (**87**), 2 μM OleD in a total volume of 100 μL. The mixture was incubated in an orbital shaker at 30 ^{o}C for 1 h. The products and the remaining **1** were extracted with 100 μL of chloroform. The organic extract was applied on TLC plates (20 μL/spot). The plates were developed with a solvent mixture of chloroform/methanol/25% NH_4OH (89:9.9:0.1), and stained with 0.75% vanilla and 1.5% H_2SO_4 in methanol.

Alternatively, to assess the substrate specificity of OleD, **87** was replaced with UDP-D-galactose (**88**), UDP-D-mannose (**89**), UDP-D-xylose (**90**), UDP-D-glucuronic acid (**91**), UDP-D-*N*-acetylglucosamine (**92**), TDP-D-glucose, CDP-D-glucose, or GDP-D-glucose, and **1** was replaced with erythromycin A (**6**, as a hydrate), oleandomycin (**9**, as a phosphate salt), or tylosin (**13**, as a tartrate). All of the above were purchased from Sigma-Aldrich.

3.2.6 Coupled E_p/OleD Assay

The sample of *Salmonella enterica* LT2 α-D-glucopyranosyl phosphate thymidylyltransferase (E_p) was provided by Professor Jon Thorson currently of

the University of Wisconsin.[17] The stock of the E_p enzyme has the activity of 7.04 U/μL, where a unit of activity (U) is defined as the amount of the protein needed to produce 1 μmol of TDP-D-glucose per min. D-Glucose-1-phosphate (**93**) and 2-deoxy-2-amino-D-glucose-1-phosphate (**98**) were purchased from Sigma-Aldrich. The synthetic samples of 6-deoxy-D-glucose-1-phosphate (**94**), 4-deoxy-D-glucose-1-phosphate (**95**), 3-deoxy-D-glucose-1-phosphate (**96**), 2-deoxy-D-glucose-1-phosphate (**97**), and 4,6-dideoxy-4-amino-D-glucose-1-phosphate (**99**) were provided by Professor Jon Thorson. The E_p reaction was conducted first. The reaction mixture consisted of 50 mM sodium phosphate (pH 7.5), 5.5 mM $MgCl_2$, 10 mM sugar-1-phosphate, 5 mM UTP, and 0.5 μL (3.52 U) of E_p in a total volume of 50 μL. The mixture was incubated in an orbital shaker at 37 ^oC for 30 min. It was then chilled on ice and the components of the OleD reaction were added to bring the concentrations to 50 mM sodium phosphate (pH 7.5), 5.5 mM $MgCl_2$, 1 mM tylosin (**13**), and 2.1 μM (0.2 nmol) OleD (5 μL of 2 mg/mL stock) in a final volume of 100 μL. The reaction mixture was incubated at 30 ^oC for 1 h and the products were analyzed by TLC as described in Section 3.2.5.

3.2.7 Screening *S. venezuelae* Genomic DNA Library for the MGT Gene

The *oleD* gene amplified for the construction of the pOleD-1 plasmid (1.3 kb) was used in the colony hybridization and the Southern hybridization experiments. The preparation of the ^{32}P-labeled *oleD* probe was performed using the Multiprime DNA Labeling System (Amersham Biosciences) according to the manufacturer's instructions. The construction of the cosmid library of the

S. venezuelae genomic DNA was carried out according to Tuan *et al.*[6] using the Packagene Kit from Promega. Dr. Lishan Zhao of our group fractionated the *S. venezuelae* chromosomal DNA partially digested with *Sau*3AI. The fragments of approximately 35 kb in length were ligated into the cosmid vector pNJ1 at the *Bgl*II sites. The ligation mixture was packaged into a λ phage and used to transfect *E. coli* LE392. The colonies were lifted onto the Biotrace NT nitrocellulose discs (Pall Life Sciences, East Hills, NY; formerly Gelman Sciences). The colony hybridization was carried out according to the protocol recommended by Pall Life Sciences. The preparation of the *S. venezuelae* genomic DNA and standard Southern hybridization protocols followed the procedures described by Hopwood *et al.*[12]

The DNA sequencing was performed by the Advanced Genetic Analysis Center of the University of Minnesota at St. Paul and the DNA Core Facility at the Institute of Cellular and Molecular Biology of the University of Texas at Austin. DNA primers for sequencing were custom-prepared by Invitrogen. The sequence data was assembled and analyzed using software packages GeneWorks (Oxford Molecular Group, San Diego, CA) and Vector NTI (Informax, Bethesda, MD). DNA and protein database searches were routinely performed using BLAST method of the National Center of Biological Information (www.ncbi.nlm.nih.gov).

3.2.8 Amplification of the 3' End of *desG* Using RACE Kit

To determine the missing sequence of the 3' end of the *oleD* homologue in *S. venezuelae*, *desG*, the RACE method was used. The 3' RACE kit was

purchased from Roche Molecular Biochemicals (Indianapolis, IN). All the procedures were carried out according to the protocol recommended by Roche Molecular Biochemicals. Total RNA of *S. venezuelae* was isolated according to the procedure described by Kieser *et al.*[13] All media, buffers, and glassware used while dealing with RNA were autoclaved twice to remove traces of any RNases. New bottles of organic solvents and plastic ware certified as RNase-free were used. The quality of the RNA preparations was assessed using spectrophotometry and agarose gel electrophoresis.[13]

The first strand of cDNA was synthesized by the AMV reverse transcriptase from an mRNA template using the universal oligo dT-anchor primer. The total RNA of *S. venezuelae* was used as the source of the mRNA template. The cDNA was in turn used as the template for the PCR synthesis of *desG* by the *pfu* DNA polymerase (Stratagene, La Jolla, CA), using the sequence specific primer SP1 (forward, 5'-CTAGGGTGTCCGCCATGAC-3') and the PCR anchor primer (reverse). The attempts to clone the PCR product (1.8 kb) in the pGEM vector (Promega) using the A-tailing procedure recommended by the pGEM vector's manufacturer failed.

To create the restriction sites for cloning of the PCR product, we amplified the fragment of the cDNA using primers desGseq-6 (5′-GCGCAAGCTT-AGG**GTG**TCCGCCATGAC-3′, corresponding to the beginning of *desG* with the potential GTG start codon shown in bold) and desGseq-8 (5′-GGCCGAATTC-CACGCGTATCGATGTCGAC-3′, which is the same as the PCR anchor primer with the restriction site added) with engineered *Hind*III and *Eco*RI sites

(underlined), respectively. The PCR product of approximately 1.8 kb was cloned at the *Hind*III-*Eco*RI sites of the pUC119 vector, and the insert of the resulting plasmid was sequenced using primers from Promega, M13/pUC-forward and M13/pUC-reverse, corresponding to the portions of the pUC119 vector. No *oleD* homologues were found.

3.2.9 PCR Amplification of *desG* Using Degenerated Primers

The degenerated reverse primer was designed based on the alignment of the protein sequences of the homologous macrolide glycosyltransferases. The protein sequence DLIEAEL, located at the *C*-terminal portion of macrolide glycosyltransferases, was used for designing the primer. Variable bases were introduced in the DNA sequence of the primer to match the codon preferences for *Streptomyces*.[13] The resulting primer, desGseq-14, had the sequence 5′-GGCC-GAATTCSAGYTCSGCYTCGATSAGGTC-3′, where the restriction site for *Eco*RI is underlined, S = C + G, and Y = C + T.

The fragment containing *desG* was amplified by PCR using primers desGseq-6 and desGseq-14, and the pSB-Sv-0301 cosmid containing the entire *desG* gene as the template. The PCR product (1.2 kb) was cloned in the *Hind*III-*Eco*RI sites of the pUC119 vector to give the plasmid pDesG0614. The insert of pDesG0614 was sequenced using primers M13/pUC-forward and M13/pUC-reverse. The obtained nucleotide sequence was determined to be a part of *desG*. It was used to design primers for sequencing the region of *desG* further downstream: desGseq-15 (5′-GGATCCAGGCGGACATGGCG-3′) and desGseq-

16 (5′-CTGCCGATGGAGAACGTCACC-3′). The resulting sequencing information allowed the completion of the *desG* sequence (Figure 3-9).

3.2.10 DesG: Expression, Purification, and Activity Assay

Construction of the Expression Plasmids pDesG-C-His and pDesG-N-His. The *desG* gene was expressed as two recombinant proteins, DesG-C-His and DesG-N-His, containing a *C*-terminal and an *N*-terminal His_6 tag, respectively. For the expression as DesG-C-His, the *desG* gene was amplified by PCR using the following primers: forward primer 5'-GCCGCATATGACCCCCGCTCC-3', containing an *Nde*I restriction site (underlined) and reverse primer 5'-GCGC-AAGCTTGCCGCGCCGCGGGCCTCGGTCGAGCTCG-3' containing a *Hind*III restriction site (underlined). For the expression as DesG-N-His, the *desG* gene was amplified by PCR using the same forward primer and the reverse primer 5'-GCGCAAGCTTCAGCCGCGCCGCGGGCCTCGGTCGAGCTCG-3' containing a *Hind*III restriction site (underlined). The cosmid pSB-Sv-0301 was used as the template. The DNA fragments were amplified by PCR and cloned into the *Nde*I-*Hind*III sites of the vector pET24b(+) to give pDesG-C-His, or the same sites of the vector pET28b(+) to give pDesG-N-His. These plasmids were used to transform *E. coli* BL21(DE3).

Cell Growth. An overnight culture of *E. coli* BL21(DE3)/pDesG-C-His or *E. coli* BL21(DE3)/pDesG-N-His was used to inoculate 1 L cultures (1% v/v) of the LB medium containing kanamycin (35 μg/mL). The cultures were incubated at 37 ^{o}C until the OD_{600} reached 0.5. The temperature of incubation was lowered

to 18 ^{o}C and the cultures were induced with 0.3 mM IPTG. After additional 16 h incubation at 18 ^{o}C, the cells were harvested by centrifugation (5,000 g, 15 min).

Crude Extract Preparation. Cell pellet from 3 L of culture was re-suspended in 55 mL of the lysis buffer (50 mM potassium phosphate, 0.2% (v/v) Triton X-100, pH 7.5) containing 0.1 mg/mL of lysozyme and incubated on ice for 30 min. The cells were disrupted by sonication in thirty 10-sec bursts with a 20-sec cooling period between each blast. Cell debris was removed by centrifugation (12,000 g, 20 min), and the supernatant was used in the next step.

Affinity Purification Using Ni-NTA Resin. The supernatant was mixed by a slow agitation with 6 mL of the packed Ni-NTA agarose resin (pre-washed in the lysis buffer) for 2.5 h at 4 ^{o}C. The slurry was poured into a capped column and washed with 10 mL of a lysis buffer. It was followed by washing with 20 mL of the lysis buffer that contained 10 mM imidazole and 30 mL of the lysis buffer that contained 30 mM imidazole. The target protein was eluted with the lysis buffer containing 250 mM imidazole. The desired fractions, as detected by SDS-PAGE, were pooled and dialyzed against two 2 L changes of the 50 mM potassium phosphate buffer (pH 7.5) containing 10% glycerol. The protein samples were stored at –80 ^{o}C. The SDS-PAGE of DesG-N-His and DesG-C-His purified using the Ni-NTA resin is shown in Figure 3-2, lanes 1 and 2, respectively.

Affinity Chromatography Using UDP-agarose Resin. Attempts were also made to further purify DesG-N-His using the UDP-agarose resin. Two published

procedures were tried,[18, 19] but DesG-N-His did not bind to the resin under these conditions. This technique was therefore abandoned.

DesG Activity Assay. The glycosyltransferase activity of DesG was determined by the TLC assay. The assay mixture consisted of 50 mM Tris-HCl (pH 8), 5 mM $MgCl_2$, 0.5 mM methymycin (**1**) or tylosin (**13**), 2 mM UDP-glucose (**87**), 20 μL of DesG-C-His or DesG-N-His (approximately 2.5 mg/mL, 0.5 μM), in a total volume of 100 μL. The mixture was incubated in an orbital shaker at 30 ^{o}C for 1 h. The products and the remaining aglycone were extracted with 100 μL of chloroform and analyzed by TLC as described in Section 3.2.5.

3.3 RESULTS AND DISCUSSION

If the proposed glycosylation-deglycosylation model is the primary self-resistance mechanism for *S. venezuelae*, one would expect **85** and **86** be the major products produced by the KdesR mutant. However, contrary to this prediction, both compounds were found only as minor products in the fermentation broth of the *desR* mutant.[2] Further experiments showed that there is no second copy of *desR* or other *desR* homologue capable of hydrolyzing the diglycosides produced in the *desR* disrupted mutants.[5] Thus, a possible reason for the low yields of **85** and **86** may be due to the low expression level of the macrolide glycosyl-transferase.

3.3.1 Increasing Production of Glucosylated Macrolides by Expression of *oleD* in KdesR Mutant

The yield of the glucosylated macrolides may be increased if the responsible glucosyltransferase is overproduced, or an exogenous MGT, which

has an identical function, is introduced into the *desR* disrupted mutants. Because the glucosyltransferase catalyzing the post-synthesis modification in *S. venezuelae* has not been found, we decided to use OleD instead. This enzyme, along with OleI, are the two macrolide glycosyltransferases involved in the self-resistance mechanism in the oleandomycin producer *S. antibioticus*.[20] Both catalyze the β-glucosylation at the C-2′ hydroxyl of the desosamine moiety. The *oleI* gene is located within the oleandomycin biosynthetic cluster, and the encoded OleI is specific for oleandomycin. In contrast, *oleD* is not a part of the oleandomycin cluster, and its translated product, OleD, is capable of glycosylating a number of macrolide antibiotics including oleandomycin (**9**), erythromycin (**6**), carbomycin (**14**) and tylosin (**13**).[20]

In order to express the *oleD* gene in *S. venezuelae,* it was amplified by PCR from the genomic DNA of *S. antibioticus* and cloned into pDHS617 at the *Eco*RI-*Xba*I sites to give the plasmid pOleD-1. The vector pDHS617 is derived from pOJ446,[7] and it contains a promoter sequence from the methymycin/pikromycin cluster[1] that enables the expression of foreign genes in *S. venezuelae*.[8] The plasmid pOleD-1 was introduced into KdesR-11 by conjugal transfer using *E. coli* S17-1, and the pOleD-1-containing strains were selected on the basis of their thiostrepton resistant ($Thio^R$) and apramycin resistant (Apr^R) phenotypes. The examination of the macrolide production using TLC showed that the amounts of glucosylated methymycin (**85**) and glucosylated neomethymycin (**86**) made by oleD/KdesR strain were approximately eight times

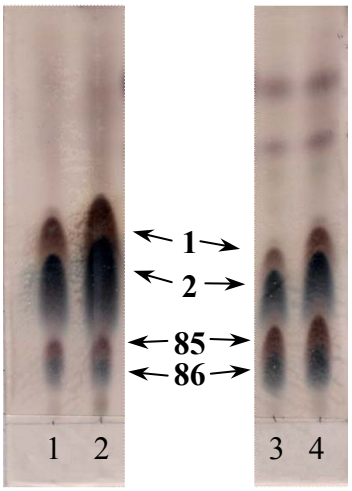

Figure 3-3: TLC analysis of the macrolides produced by *S. venezuelae* KdesR (lanes 1 and 2) and *S. venezuelae* oleD/KdesR (lanes 3 and 4). Methymycin (**1**), neomethymycin (**2**), glucosylated methymycin (**85**) and glucosylated neomethymycin (**86**) are labeled.

more than those made by the KdesR mutants (Figure 3-3). Up to 40% of methymycin and neomethymycin produced are now C-2' glucosylated.

3.3.2 Deletion of the *pikR1* and *pikR2* Genes in *S. venezuelae* KdesR

Since **1** and **2** are still the major products in the fermentation broth of the oleD/KdesR mutant, glucosylation of the macrolide products to give **85** and **86** is unlikely the primary self-resistance mechanism in *S. venezuelae*. Instead, the two MLS_B type resistant genes, *pikR1* and *pikR2*, found upstream of the PKS (Figure 1-15) may provide the major cellular self-protection for *S. venezuelae*. The reason that two of these genes exist may be related to the fact that two distinct polyketide ring systems (**1**/**2** and **4**/**5**) are produced by this strain. A disruption of both genes might force the bacterium to rely on the secondary self-protection mechanism. Thus, it is conceivable that a triple mutant in which *pikR1*, *pikR2* and

desR genes are disrupted or deleted may lead to the accumulation of **85** and **86** as major products. On the basis of this premise, a disruption plasmid, pPikR-K, was constructed, in which most of *pikR1* and *pikR2* sequences were deleted and replaced by the kanamycin resistance gene. The plasmid pPikR-K was introduced into the KdesR-11 mutant, followed by the screening of the double-crossover mutants. However, all attempts to obtain mutants with a phenotype of apramycin sensitive (Apr^S), thiostrepton resistant ($Thio^R$), and kanamycin resistant (Kan^R) failed. This observation suggests that the methylation of an adenine residue in the 23S RNA mediated by PikR1/PikR2 likely confers the primary cellular self-protection to *S. venezuelae*, and *S. venezuelae* cannot survive without *pikR1* and *pikR2*.

3.3.3 Purification and Substrate Specificity of the Macrolide Glycosyltransferase OleD

Macrolide glycosyltransferases can potentially be used as a tool for the post-synthesis modification of macrolides. The existing sugar substituent may be further derivatized by an MGT to form a disaccharide chain. If this MGT is flexible enough, a variety of disaccharides can be generated, some of which may confer enhanced or diverged biological activities to the parent macrolide. Alternatively, an MGT may be useful for *in vivo* glucosylation of genetically engineered "unnatural" macrolides. Such a modification may prevent the inhibition of the growth or death of the bacteria producer incurred by the new compounds if they are antibiotics and glycosylation catalyzed by MGT leads to inactivation of their activities. The active macrolide can then be regenerated by the action of the β-glycosidase *in vitro*. In view of these potentials, we decided to

first probe the substrate specificity of the MGT *in vitro*. Since the MGT of *S. venezuelae* was not available, we expressed and purified the MGT of *S. antibioticus*, OleD. We then carried out the preliminary testing of its substrate specificity using commercially available macrolides and sugar nucleotides. The coupled E_p/OleD assay was also developed, allowing the glycosylation of macrolides with sugar-1-phosphates directly.

Overexpression of oleD and Purification of the Recombinant Protein. In order to purify the OleD protein, the *oleD* gene was cloned into the expression vector pET24b(+) to produce *C*-terminal His_6-tagged fusion protein in *E. coli*. The resulting plasmid, pOleD-2, was used to transform *E. coli* BL21(DE3) competent cells. The induction of *E. coli* BL21(DE3)/pOleD-2 with IPTG resulted in the expression of OleD. The desired protein was purified by affinity chromatography on the Ni-NTA column, followed by the anion exchange chromatography on a DEAE column (Figure 3-2, lane 3). The purified protein was used in the activity assays.

Testing Substrate Specificity of OleD Using Sugar Nucleotides. The capability of the recombinant OleD to glycosylate various macrolides using different sugar nucleotides was investigated using commercially available substrates. The structures of the sugar nucleotides tested in this experiment are shown in Figure 3.4. The extent of the glycosylation was monitored by TLC. The products having an R_f value much lower than those of the unmodified macrolides are likely being further glycosylated. Table 3-1 summarizes the results of this experiment. The approximate value for the yield of a glycosylated

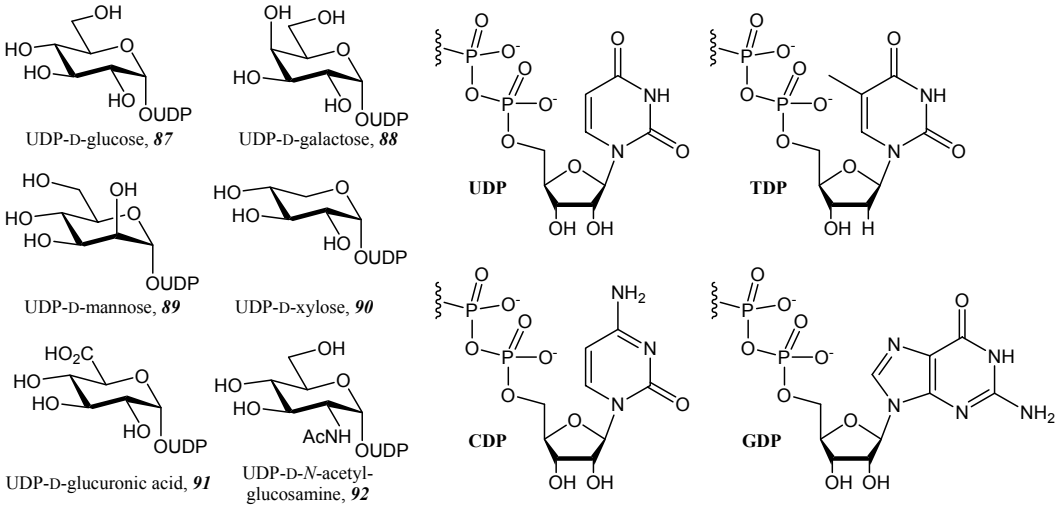

Figure 3-4: The structures of sugar nucleotides used in the OleD assay.

product (% of the total macrolides present as a glycosylated product) was estimated based on the intensity of the TLC spots.

It has been reported that OleD can efficiently glycosylate oleandomycin (**9**), erythromycin (**6**), tylosin (**13**), and carbomycin (**14**) using UDP-α-D-glucose (**87**) as the sugar donor.[20] Our experimental results are in an agreement with this report, since OleD demonstrated the full conversion of **6**, **9**, and **13** to their glycosylated derivatives in the assay with **87** (entries 10, 12, and 14, respectively). Furthermore, we found that methymycin (**1**) was glycosylated by OleD with a lower but significant efficiency (entry 1). This data demonstrates the flexibility of OleD toward the acceptor substrate, consistent with its involvement in a general resistance mechanism, not a self-resistance one.[20]

As expected, OleD showed a strong preference for UDP-D-glucose (**87**) over other nucleotides tested (entry 1 vs. entries 7, 8, and 9). TDP-D-glucose was a poor substrate, resulting in more than a 3-fold decrease in the yield of

Table 3-1: Summary of the Glycosylation Reactions Catalyzed by OleD

Entry	Sugar Nucleotide Substrate	Macrolide Substrate	Estimated % Conversion
1	UDP-D-glucose (**87**)	**1**	70
2	UDP-D-galactose (**88**)	**1**	NP[a]
3	UDP-D-mannose (**89**)	**1**	NP
4	UDP-D-xylose (**90**)	**1**	40
5	UDP-D-glucuronic acid (**91**)	**1**	NP
6	UDP-D-*N*-acetylglucosamine (**92**)	**1**	NP
7	TDP-D-glucose	**1**	20
8	CDP-D-glucose	**1**	5
9	GDP-D-glucose	**1**	NP
10	**87**	**6**	100
11	**90**	**6**	100
12	**87**	**9**	100
13	**90**	**9**	NP
14	**87**	**13**	100
15	**90**	**13**	60

[a] No product detected

glycosylated methymycin (entry 7), whereas CDP-D-glucose only gave trace amounts of the product (entry 8). GDP-D-glucose was not a substrate at all (entry 9).

We also tested several UDP-sugars as donor substrates for the methymycin glycosylation by OleD (entries 1-6). Only the addition of **87** or UDP-D-xylose (**90**) to the assay resulted in the production of the glycosylated methymycin. Interestingly, neither C-4 nor C-2 epimer of **87**, UDP-D-galactose (**88**) or UDP-D-mannose (**89**), respectively, was a substrate for OleD. Evidently, OleD is much less tolerant to the variation of the sugar donor structures than to those of the macrolide acceptors.

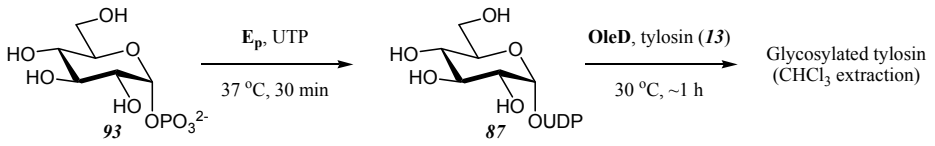

Figure 3-5: Scheme of E_p/OleD assay.

E_p/OleD Coupled Assay Using Sugar-1-phosphates. To broaden the selection of the donor substrates we designed an E_p/OleD coupled assay to make UDP-sugar directly from sugar-1-phosphate *in situ*. The *Salmonella enterica* LT2 α-D-glucopyranosyl phosphate thymidylyltransferase (E_p) can catalyze the conversion of D-glucose-1-phosphate (**93**) to UDP-D-glucose (**87**) and has been shown to possess a broad substrate specificity.[17] Several synthetic sugar-1-phosphates, as well as the stock of E_p enzyme, were kindly provided by Professor Jon Thorson of the University of Wisconsin. Dr. Thorson and his coworkers have developed the general conditions for the E_p assay.

The schematic illustration of the coupled E_p/OleD assay is shown in Figure 3-5. In this assay, sugar-1-phosphate was first converted to its UDP derivative by E_p in 50 μL of buffer. Then, tylosin (**13**) was added to this reaction, and the reaction volume was brought to 100 μL. The amounts of reagents were such that a 100% conversion by E_p, using UTP as the limiting reagent, would lead

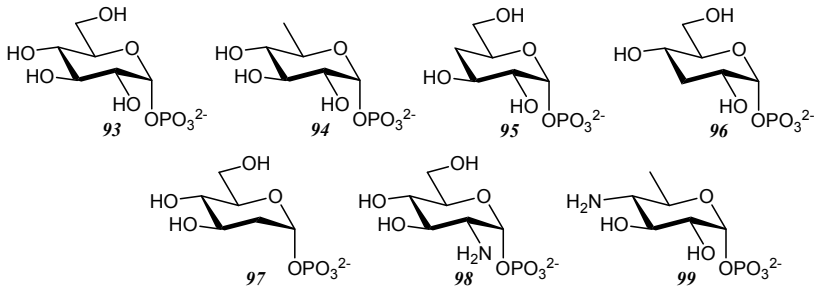

Figure 3-6: The structures of sugar-1-phosphates used in the E_p/OleD assay.

Table 3-2: Summary of the Glycosylation Reactions Catalyzed by E_p/OleD

Entry	Sugar-1-phosphate Substrate	Estimated % Conversion of Macrolide	% Conversion by E_p[a]
1	D-glucose-1-phosphate (**93**)	≥95	99.5
2	6-deoxy-D-glucose-1-phosphate (**94**)	≥95	99.1
3	4-deoxy-D-glucose-1-phosphate (**95**)	NP[b]	99.3
4	3-deoxy-D-glucose-1-phosphate (**96**)	NP	6.5
5	2-deoxy-D-glucose-1-phosphate (**97**)	~5	22.3
6	2-deoxy-2-amino-D-glucose-1-phosphate (**98**)	NP	~100
7	4,6-dideoxy-4-amino-D-glucose-1-phosphate (**99**)	NP	~50

[a] The values are as reported in the Ref. 16 (entries 1-5) or provided by Prof. Thorson through personal communications (entries 6 and 7); [b] No product detected

to a 2.5 fold excess of the UDP-sugar over tylosin. OleD was then added and, after incubation, the macrolides were extracted with chloroform and analyzed by TLC. The sugar-1-phosphates tested are shown in Figure 3-6. The results of this experiment are summarized in Table 3-2. Tylosin (**13**) was used as an acceptor in all experiments, since it is a good substrate for OleD, as was demonstrated in the previous assay.

The results obtained in this experiment showed that OleD is quite sensitive to the variations in the structure of the sugar donor. Among the compounds tested, only UDP-6-deoxy-D-glucose (derived from **94**) (entry 2) was accepted by OleD as efficiently as its natural substrate, UDP-D-glucose (derived from **93**) (entry 1). The inclusion of UDP-2-deoxy-D-glucose-1-phosphate (derived from **97**) in the assay only resulted in a very low yield of glycosylated tylosin (entry 5).

Meanwhile, incubation with UDP derivatives of sugar-1-phosphates **95**, **96**, **98**, and **99** did not give glycosylation of tylosin at all (entries 3, 4, 6, and 7, respectively). The low conversion of **96** by E_p to its UDP derivative may have limited the supply of the sugar donor. However, the failure to glycosylate tylosin by OleD in the presence of the UDP derivatives of **95**, **96**, **98**, and **99** is most likely because they are not substrates for OleD.

3.3.4 Identification and Sequencing of the Macrolide Glycosyltransferase Gene of *S. venezuelae*

The above results clearly showed that the UDP-sugar substrate specificity of the MGT OleD is quite stringent. It would be interesting to determine whether other MGTs also share a similar stringency of substrate specificity. Our next target is the MGT, designated as DesG from here on, operating in *S. venezuelae*. Its existence is implicated by the detection of diglycosylated macrolides **85** and **86** produced by the *S. venezuelae* KdesR strain. Because the corresponding gene is not a part of the methymycin/pikromycin biosynthetic cluster, it is possible that, like OleD, DesG is involved in the general resistance mechanism rather than the self-resistance mechanism. If this is the case, it may have broad substrate specificity, a property desirable for post-synthesis modification.

Screening S. venezuelae Genomic DNA Library for the MGT Gene. The cosmid library of the *S. venezuelae* genome was constructed using the cosmid pNJ1. It was packaged into the λ phage, and the phage mix was used to transfect *E. coli* LE392. A library of 1039 clones was generated. This library was screened by colony hybridization for the MGT gene using the ^{32}P labeled *oleD* probe. Three positive clones were identified. The cosmid DNA of these clones

was digested with *Bam*HI. Southern hybridization with the *oleD* probe revealed that one of them, pSB-Sv-0301, carries a gene homologous to *oleD*. The same band of 4 kb was detected by Southern blot for pSB-Sv-0301 and *Bam*HI-digested *S. venezuelae* genomic DNA.

The 4 kb *Bam*HI fragment of pSB-Sv-0301 was subcloned into pUC119 to generate the plasmid pSB-Sv-B4.0. This plasmid was digested by *Hinc*II. The resulting five fragments were ligated into pUC119 and sequenced using the primers complementary to the pUC119 sequence. The comparison of the sequencing data to the nucleotide sequences in the GenBank led to the identification of an approximately 1 kb of the gene with high sequence similarity to MGT genes, *desG*. Two putative start codons, six base pairs apart, were found, confirming that 5' terminus of *desG* has been found. However, the plasmid pSB-Sv-B4.0 is missing an estimated 200 bp of the 3'-terminal gene sequence.

Since the sequence of the ends of the pSB-Sv-0301 insert showed little homology to MGT genes, this plasmid must harbor the entire *desG* in the middle. Initially, "primer walking" was used in an attempt to sequence the 3'-terminal region of *desG*. The primers complementary to the upstream region of *desG* that had been sequenced were designed. However, the attempts to sequence the missing part of *desG* using these primers were unsuccessful. Over 40 subclones of pSB-Sv-0301 were then generated and probed with labeled *oleD*. The ends of the inserts in selected clones were sequenced. Only one of these plasmids, pSB-Sv-P20.0, was found to contain the entire *desG*. However, we had little success in

sequencing pSB-Sv-P20.0 using "primer walking". This effort only added the sequence of 50 bp to the 3'-terminus.

Using 3′ RACE to Determine 3′ Sequence of desG. The method called 3′ RACE was used to identify and isolate the *desG* gene. This method takes advantage of the natural poly(A)-tail of mRNA as a priming site for the PCR amplification. As illustrated in Figure 3-7, The first-strand cDNA synthesis is initiated at the poly(A)-tail of mRNA using the universal oligo dT-anchor primer. After converting mRNA into cDNA, the latter is used without further purification as the template for the PCR amplification using the PCR anchor primer and a designed specific primer.

Total RNA of *S. venezuelae* was isolated and its quality was assessed using spectrophotometry and agarose gel electrophoresis.[13] The A_{260}:A_{280} ratio

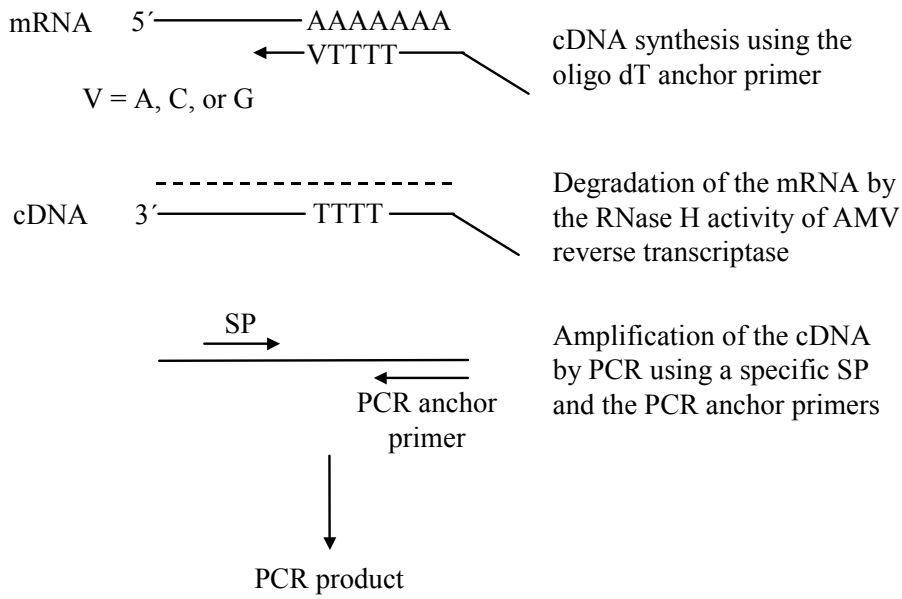

Figure 3-7: Overview of 3′ RACE. The figure is taken from the "5′/3′ RACE Kit" instruction manual by Roche Molecular Biochemicals.

for the selected RNA sample was 2.02, indicating that it is not contaminated with protein (it is above 1.9), and RNA is not degraded (values greater than 2.10 indicate severe degradation). The bands for 23S, 16S, and 5S rRNA were clearly visible on the agarose gel, indicating that RNA is not degraded. The mRNA is usually not visible on the gel due to its low abundance. The sheared chromosomal DNA was removed by the treatment with DNase. The sample of total RNA was subjected to 3′ RACE.

The 3′ RACE procedure led to the amplification of an approximately 1.8 kb fragment of DNA. This fragment was cloned into the pUC119 vector, and the insert was sequenced. To our disappointment, the sequence of the insert showed no homology to *oleD*. It appears that a nonspecific DNA product was isolated. Increasing the annealing temperature from 60 to 65 ^{o}C during the PCR amplification of the cDNA led to the production of the same 1.8 kb DNA fragment. Therefore, this method was abandoned.

PCR Amplification of desG Using Degenerated Primers. After failing to determine the 3′-terminal sequence of *desG* using 3′ RACE, we attempted to amplify *desG* using degenerated primer designed based on the sequence of the MGT genes homologous to *desG*. Using BlastX, the following macrolide resistance glycosyltransferases which have high sequence similarity to the translated known portion of *desG* were selected: OleD of *S. antibioticus* (74% sequence identity),[21] MgtA of *Streptomyces ambofaciens* (70%),[22] Mgt of *Streptomyces cœlicolor* A3(2) (70%),[23] and StrLI of *Streptomyces lividans*

```
                361                                                                 426
      Mgt (361) TLPTEEATAKALRTAALALVDDPEVAARLKEIQARMAQEGGTRRAADLIEAELAAARG
     MgtA (360) HLPTEEATAEALRAAGLALVEDPEVARRLKEIQAGMAREGGTRRAADLIEAELAAART
     OleD (346) KLATEEATADLLRETALALVDDPEVARRLRRIQAEMAQEGGTRRAADLIEAELPARHERQEPVGDR
    StrLI (361) TLPTEEATAKALRTAALALVDDPEVAARLKEIQARMAQEAGTRGPADLIEAELAAARG
Consensus (361) TLPTEEATAKALRTAALALVDDPEVARRLKEIQARMAQEGGTRRAADLIEAELAAARG
```

Figure 3-8: Protein sequence alignment of *C*-terminal portions of DesG homologues: Mgt of *Streptomyces cœlicolor* A3(2), MgtA of *Streptomyces ambofaciens*, OleD of *S. antibioticus*, and StrLI of *Streptomyces lividans*. The last 25 residues of OleD are not shown.

(69%).[24] These sequences were aligned using VectorNTI software (Figure 3-8 shows the *C*-terminal portion of the alignment).

The alignment of the translated *desG* homologues revealed the segment of DLIEAEL (shown in bold in Figure 3-8) conserved among all four MTGs. This short protein sequence was used to design the reverse primer for the *desG* amplification. Variable bases were introduced in the primer sequence to accommodate the codon preferences for *Streptomyces*.[13] The resulting primer, together with the forward primer complementary to the 5′ terminus of *desG*, were used in the PCR amplification. The pSB-Sv-0301 cosmid was used as the template. The PCR product of approximately 1.2 kb was cloned into the pUC119 vector and sequenced. To our delight, not only that sequence was indeed a part of *desG*, but it also contained an additional 150 bp of the DNA sequence at its 3′ terminus that have not been seen before. Two sequencing primers matching this new sequence were designed and used for the sequencing of the remaining portion of *desG* in the pSB-Sv-0301 cosmid. This led to the completion of the *desG* sequence.

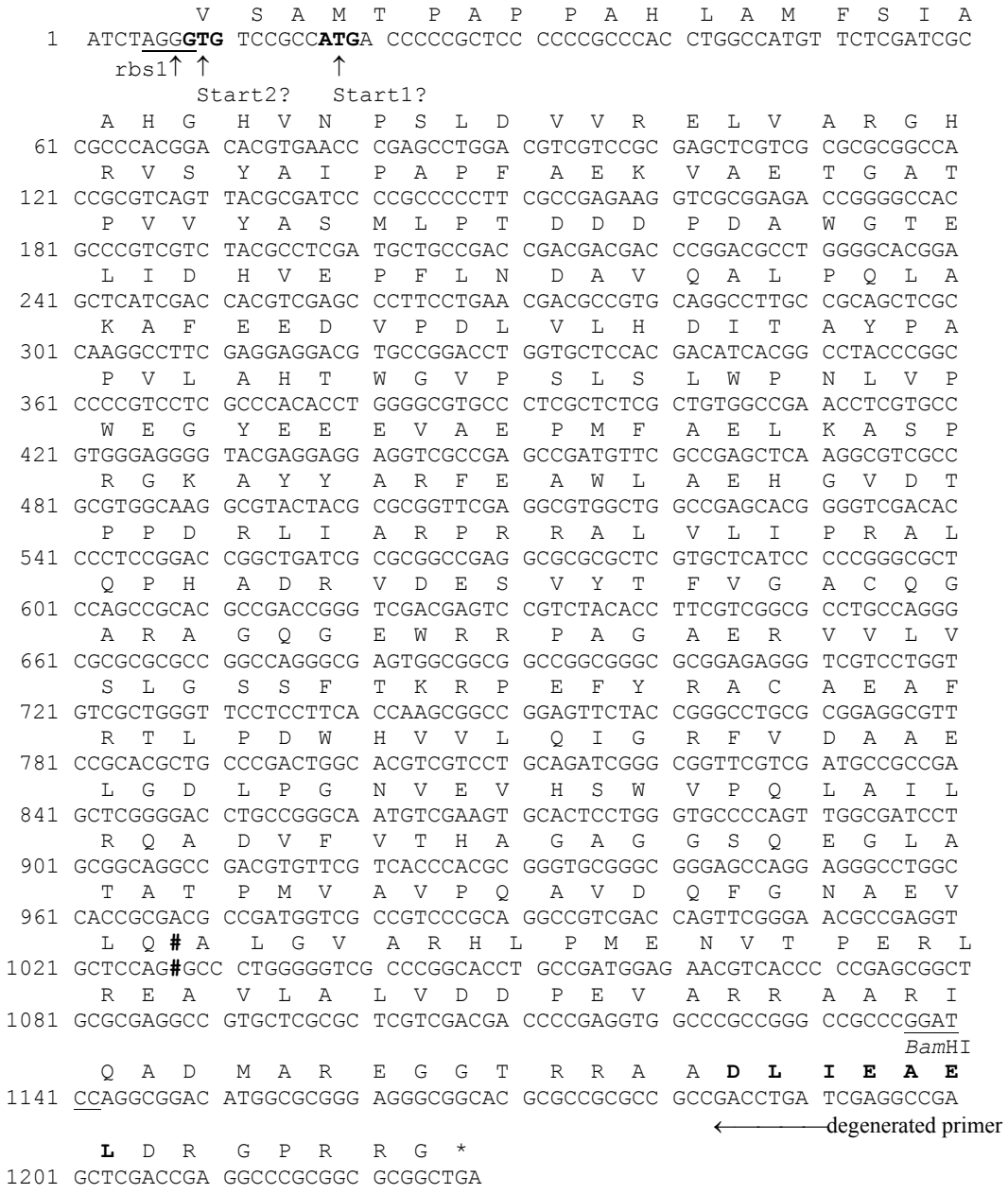

Figure 3-9: The complete nucleotide sequence of the *desG* gene and its translated protein sequence. Two possible start codons, the ribosome binding site (rbs1), and the region complementary to the degenerated primer are labeled. The beginning of the problem region of the DNA sequence is denoted by #.

The complete sequence of *desG* and its translated amino acid sequence are shown in Figure 3-9. Two possible start codons were identified: ATG (start1) and GTG (start2). Depending on which start codon is used, *desG* is either 1212 (start1) or 1221 (start2) bp long, and encodes a 403- or 406-amino acid protein. The sequence of AGGG, 8 bp upstream of start1, may serve as the potential ribosome binding site (RBS) for the translation of DesG from start1. There was no such RBS corresponding to start2. The entire translated *desG* maintains the level of sequence identity to OleD, MgtA, Mgt of *Streptomyces cœlicolor* A3(2), and StrLI.

3.3.5 Preliminary Characterization of *S. venezuelae* Macrolide Glycosyltransferase DesG

Having successfully completed the sequencing, the *desG* gene was overexpressed to determine whether the DesG protein can act as an MGT. The *desG* gene (the 1212 bp version) was cloned into pET28b(+) and pET24b(+) vectors for the expression as an *N*- and a *C*-terminal His_6-tagged protein, respectively. Both proteins, DesG-N-His and DesG-C-His, were overproduced in *E. coli* BL21 and purified using affinity chromatography on the Ni-NTA resin. DesG-N-His and DesG-C-His showed similar properties during the purification. Even though some of the protein existed as inclusion bodies after cell disruption, there was a large amount of soluble protein. The yields of DesG-N-His and DesG-C-His after the Ni-NTA column purification (Figure 3-2, lanes 1 and 2, respectively) were approximately 50–55 mg of protein per 3 L of the culture. The protein samples contained minor impurities. However, they were used without further purification for the preliminary activity assays.

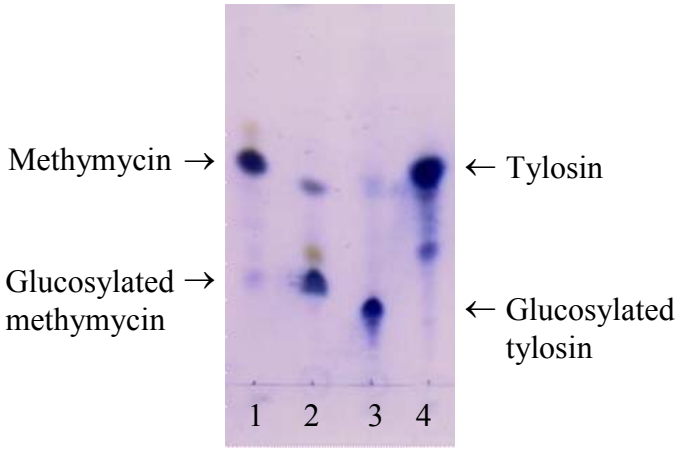

Figure 3-10: Preliminary TLC analysis of the DesG-N-His activity as a macrolide glycosyltransferase (similar results were obtained with DesG-C-His). Lanes 1 and 2 are extracts of the assays run with methymycin (**1**), and lanes 3 and 4 are those with tylosin (**13**). Lanes 1 and 4 are control reactions without enzyme. The DesG-N-His protein was added to the assay run on lanes 2 and 3.

The ability of the fusion proteins to glucosylate methymycin (**1**) and tylosin (**13**) using UDP-α-D-glucose (**87**) was tested. Analysis by TLC demonstrated that the addition of either enzyme, DesG-N-His or DesG-C-His, to the reaction mixture led to the conversion of the macrolide starting material to a more polar product (Figure 3-10). This product is most likely a glucosylated methymycin or tylosin in the respective assays. It will be necessary to perform a large-scale reaction and fully characterize these glucosylated products. It will also be interesting to test various sugar nucleotides and macrolides in the DesG assay in order to compare its substrate preferences to those of OleD.

3.4 CONCLUSIONS

The strategy of glycosylation-deglycosylation discussed in this chapter may not be the primary self-resistance mechanism operating in *S. venezuelae*, since the glucosylated methymycin and neomethymycin are produced in significantly less quantities than their unmodified parent compounds in KdesR mutants.[2, 3] The introduction of a copy of the MGT gene from *S. antibioticus*, *oleD*, into the *desR* mutant of *S. venezuelae* led to the increase in the production of glucosylated macrolides. However, more than half of the macrolides remained unglycosylated. The mutant of *S. venezuelae* with *pikR1*, *pikR2*, and *desR* genes deleted could not be isolated. This is most likely because the deletion of *pikR1* and *pikR2* is lethal to the strain. Taken together, these results suggest that methylation of rRNA by PikR1 and PikR2 is the primary self-resistance mechanism for *S. venezuelae*.

The β-glucosidase, DesR, responsible for the deglycosylation step has been identified, since the encoding gene is in the desosamine gene cluster. The catalytic activity of DesR has been demonstrated by *in vitro* assays.[3] However, to study the corresponding MGT in *S. venezuelae* responsible for the glycosylation step has been much more challenging. In order to study the general properties of MGT, we have expressed and purified the OleD protein and chracterized its substrate specificity *in vitro*. To compensate for the small selection of the commercially available sugar nucleotides, the coupled E_p/OleD assay was developed, allowing the use of synthetic sugar-1-phosphates as sugar donors for OleD. It was found that although OleD can recognize a variety of structurally

diverse acceptors, it is considerably more stringent towards the sugar nucleotide donor.

Finally, we screened the genome of *S. venezuelae* and identified the MGT gene, *desG*, involved in the glucosylation of methymycin and neomethymycin. This gene was expressed in *E. coli*, and the catalytic activity of the purified polypeptide DesG as a macrolide glycosyltransferase was confirmed. Additional experiments are necessary to fully assess the substrate specificity of DesG. The DesG enzyme may prove to be useful as a tool for the incorporation of "unnatural" sugar moieties into monoglycosylated macrolides. This may potentially improve the biological activity of new compounds. Alternatively, DesG, together with DesR, may be used for the selective inactivation-reactivation of macrolide antibiotics.

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Chapter 4: Study of the Glycosyl Transfer by DesVII and DesVIII

4.1 INTRODUCTION

Carbohydrates play an essential role in a vast array of biological processes. It is, therefore, not surprising that glycosylating enzymes, glycosyltransferases, are abundant in all living organisms. They catalyze the transfer of carbohydrate moieties from activated donor substrates, usually nucleotide-sugars, onto a wide variety of acceptors, such as other sugars, proteins, nucleic acids, lipids, polyketides, and nonribosomally synthesized peptides, through the formation of *O*-, *N*-, or *C*- linkages. Of particular interest to our laboratory are the glycosyltransferases in prokaryotes that modulate the activity of macrolide antibiotics.

The biological activity and potency of many secondary metabolites are dependent on the composition of carbohydrate constituents of these compounds. Several comprehensive reviews summarize the role that carbohydrates play in biologically active bacterial metabolites.[1-3] It is now clear that altering the composition of sugar appendages in glycoconjugates is a promising strategy for creating new products with novel or enhanced biological activities. Hence, efforts to develop effective glycosylation strategies have been vigorously pursued by scientists for decades. Significant progress has been made recently using genetic and biochemical methods to perform glycosylation *in vivo*. As highlighted by the results of the studies described in Chapter 2, as well as numerous studies

published by our and other groups, many glycosyltransferases have been shown to possess relaxed substrate specificity and be able to couple diverse sugar and aglycone structures *in vivo* (for relevant recent reviews, see references 3-7). However, the yields are generally low, and product isolation from the fermentation broth is tedious. Moreover, such an *in vivo* approach has limited general applicability since the production of antimicrobial agents would cause inhibition of cell growth or cell death. One potential solution to overcome these hurdles is to carry out the glycosylation step *in vitro* using isolated glycosyltransferases. The *in vitro* approach would also be facilitated by using genetically engineered glycosyltransferases with the desired substrate specificity.

Although many putative glycosyltransferase genes have been identified in gene clusters encoding the biosynthetic pathways to glycosylated secondary metabolites, few of the corresponding gene products have been purified and their catalytic roles verified. In particular, examples of characterized glycosyltransferases involved in the biosynthesis of antibiotics are scarce with GtfA, GtfB, GtfC, GtfD, GtfE, and NovM, which are found in the vancomycin (**100**), chloroeremomycin (**101**),[8-10] and novobiocin (**102**) pathways,[11] as the only known examples. Both **100** and **101** are nonribosomal peptide based antibiotics, while **102** is an aromatic polyketide derived antibiotic (Figure 4-1). It should be noted that a few glycosyltransferases responsible for resistance and self-resistance to macrolide antibiotics have also been characterized *in vitro*, among which OleD and OleI from the oleandomycin (**9**) producer *Streptomyces antibioticus* are the most comprehensively studied.[12, 13] However, in these cases the enzymes are not

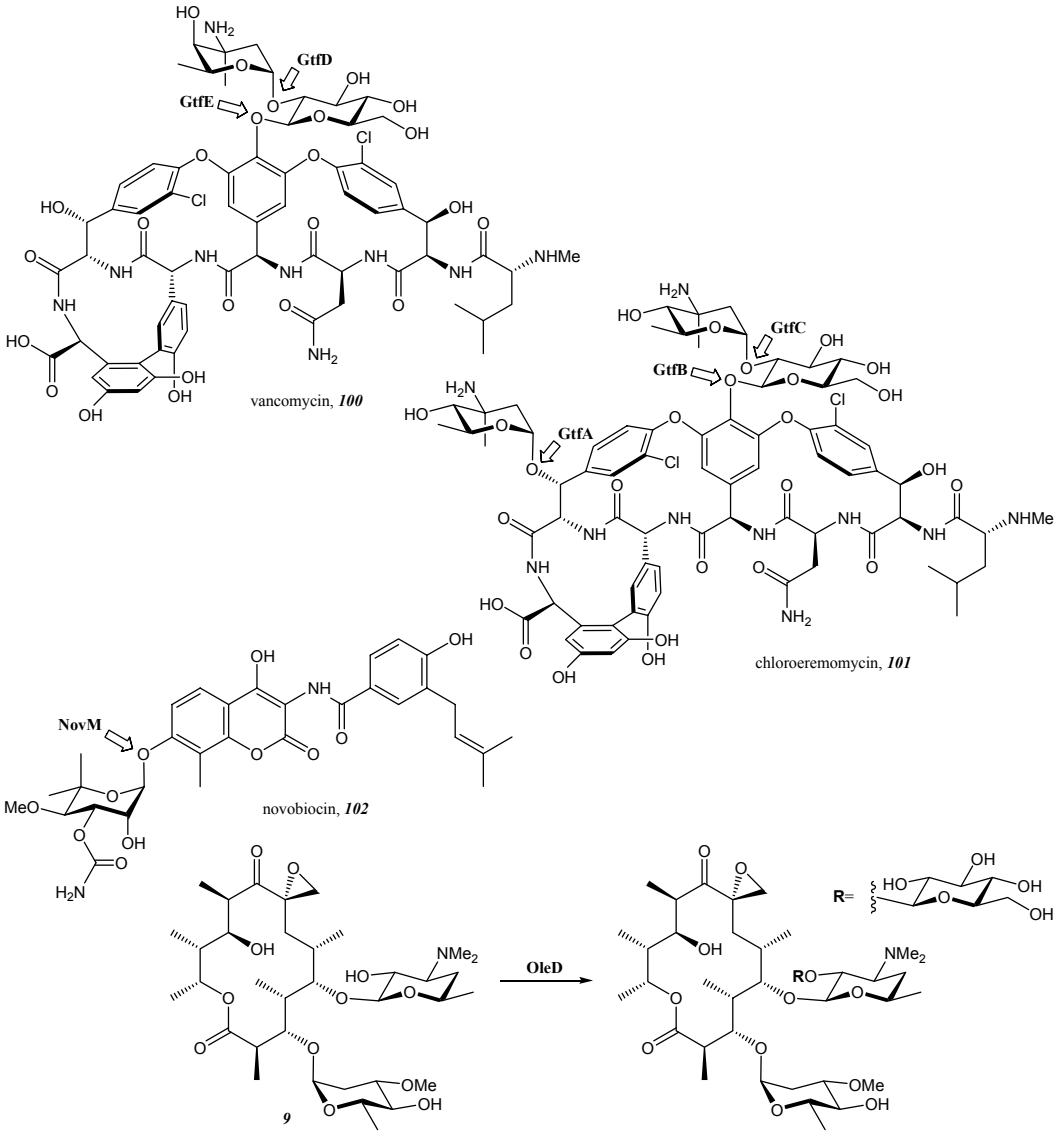

Figure 4-1: Examples of characterized glycosyltransferases involved in the biosynthesis of and resistance to secondary metabolites.

a part of a biosynthetic pathway. Instead, they catalyze the attachment of a glucose to the sugar appendages of macrolides, not the aglycone directly (Figure 4-1). Prior to this thesis research, there was no published report on *in vitro*

characterization of a glycosyltransferase participating in the biosynthesis of a macrolide. Clearly, it is important to learn the specifics of the macrolactone glycosylation. There is also an obvious need for catalysts capable of glycosylating new macrolide aglycones generated *via* manipulation of polyketide biosynthetic pathways.

This chapter describes our efforts to purify and biochemically characterize glycosyltransferase DesVII of *Streptomyces venezuelae* involved in the biosynthesis of macrolide antibiotics methymycin (**1**), neomethymycin (**2**), pikromycin (**4**), and narbomycin (**5**) (Figure 4-2). Initial attempts were made by a former group member, Dr. Lishan Zhao, to detect the desosaminyltransferase activity in the crude extracts of *Escherichia coli* expressing *desVII* as *C*-terminal His_6-tagged fusion protein. Dr. Zhao tested a range of glycosyltransferase assay conditions but failed to demonstrate the formation of the expected product,

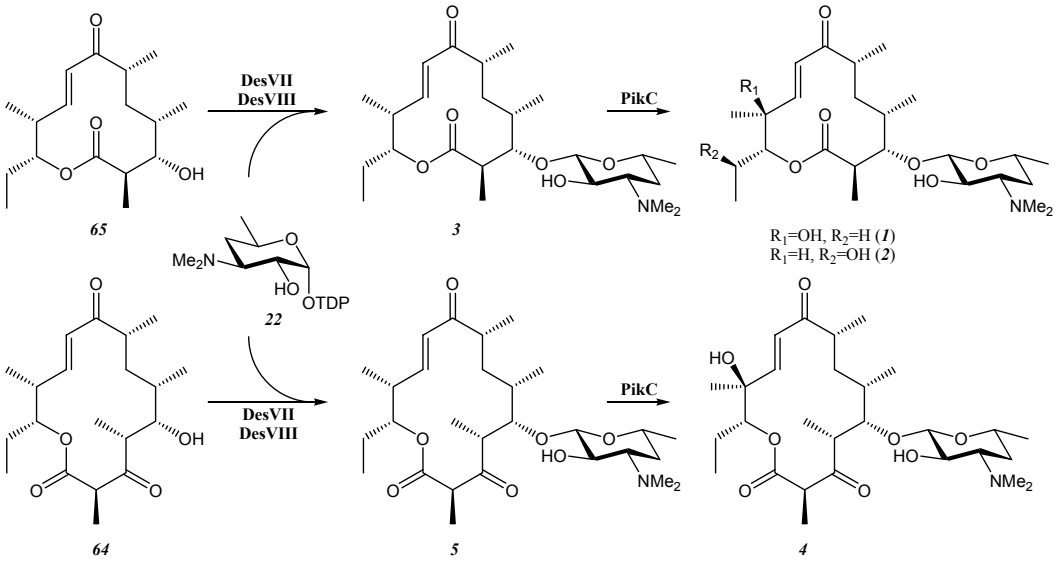

Figure 4-2: The function of macrolide glycosyltransferase DesVII described in this study.

desosaminylated 10-deoxymethynolide (**3**, YC-17, or 10-deoxymethymycin). In this study we have achieved the purification of DesVII. We have also developed effective assay conditions for its activity. The most important discovery for this glycosyltransferase is that it is active only at high pH (approximately 9) and in the presence of another protein component, DesVIII. The study resulted in the first report of a demonstrated macrolide glycosyltransferase activity *in vitro*.[14] To learn more about the catalytic properties of the DesVII/DesVIII pair, we have also tested the specificity of this enzyme pair towards various donor and acceptor substrates, mainly the intermediates of the biosynthesis of tylosin (**13**) and erythromycin (**6**).

4.2 EXPERIMENTAL PROCEDURES

4.2.1 General

Bacterial Strains. *Escherichia coli* DH5α from Invitrogen (Carlsbad, CA) was used throughout the study as the regular cloning host. *E. coli* S17-1[15] was the donor strain for conjugal transfer to *S. venezuelae*. The strains used as hosts for gene overexpression include *E. coli* BL21(DE3) and *E. coli* NovaBlue(DE3) from Novagen Inc. (Madison, WI), and *E. coli* BL21(DE3)pLysS and *E. coli* BL21-CodonPlus (DE3) from Stratagene (La Jolla, CA). Strains *S. venezuelae* ATCC 15439, *S. antibioticus* ATCC 11891, *S. lividans*/pIJ702 ATCC 35287, and *S. lividans* TK64 ATCC 69441 were obtained from American Type Culture Collection (Rockville, MD) as freeze-dried pellet and were revived according to the instructions provided by ATCC.

Plasmids, Vectors, and Biomaterials. Cosmid pLZ4 (derivative of pNJ1 vector)[16] containing desosamine biosynthetic cluster and part of polyketide synthase cluster[17, 18] was used as a template for polymerase chain reaction (PCR) to obtain appropriate DNA fragments. Plasmid pKC1139, used for conjugal transfer of DNA to *S. venezuelae*, was a gift from Dr. Leonard Katz of Abbott Laboratories.[19] Plasmid pDHS5000, which contains the *tsr* gene, was a gift from Professor David Sherman of the University of Michigan. Expression vectors pET24b(+) and pET28b(+) were from Novagen. *Pfu* DNA polymerase used for most of the PCR reactions was purchased from Stratagene. The Advantage cDNA polymerase was from Clontech (Palo Alto, CA).

Enzymes used in the cloning experiments, such as restriction endonucleases and T4 DNA ligase, the corresponding buffers, and DNA molecular weight marker were products of Invitrogen. QIAprep and QIAquick DNA purification kits from Qiagen (Valencia, CA) were used for plasmid DNA minipreps and purification of PCR products, respectively. Rapid Translation System (RTS 500) product of Roche Molecular Biochemicals (Indianapolis, IN). The procedures for *in vitro* transcription/ translation recommended by the manufacturer's manual were closely followed.

The DNA sequencing was performed by the DNA Core Facility at the Institute of Cellular and Molecular Biology of the University of Texas at Austin. DNA primers for PCR amplification and sequencing were custom-prepared by the DNA Core Facility or by Integrated DNA Technologies (IDT, Coralville, IA).

The general methods and protocols for recombinant DNA manipulations described by Sambrook *et al*.,[20] and those dealing with *Streptomyces* strains described by Hopwood *et al*.[21] and Kieser *et al*.[22] were followed in this study.

Antibiotics and most chemicals used in this study are products of Sigma-Aldrich (St. Louis, MO), VWR International (West Chester, PA), and Fisher Scientific (Pittsburgh, PA). Cytochrome P450 enzymes were obtained from Sigma-Aldrich. Radiolabeled [acetyl-^{3}H] coenzyme A was purchased from ICN Biomedicals, Inc. (Irvine, CA). Culture and media components were from BD Diagnostics System (Sparks, MD).

Ni-NTA resin for affinity purification of His-tagged proteins was obtained from Qiagen. DEAE Sepharose CL-6B resin for ion exchange and Sephacryl S200 HR resin for size exclusion protein chromatography were from Amersham Biosciences (Piscataway, NJ). Amylose affinity matrix used for purification of maltose binding protein and its fusion proteins were from New England Biolabs (Beverly, MA). Protein purification and native molecular mass determination was performed using FPLC (Amersham Biosciences) equipped with ion exchange MonoQ HR 10/10 or size exclusion Superdex 200 HR 10/30 column. Protein purification steps were carried out at 4 ^{o}C except for FPLC chromatography that was performed at room temperature.

Pre-stained protein marker was obtained from New England Biolabs. Mini-PROTEAN II vertical system used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), Mini trans-blot assembly apparatus used for Western blot, GelAir drying system used to dry gels, and the

necessary accessories and reagents were all products of BioRad. The nitrocellulose Hybond-C Extra membrane used for Western blot was from Amersham Biosciences. The antibodies monoclonal anti-polyhistidine clone His-1 and anti-mouse IgG alkaline phosphatase conjugate were purchased from Sigma. For Western blot detection, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) were both obtained from Promega.

Instrumentation. HPLC applications on enzyme assays and purification of incubation products were performed on a Beckman Coulter (Fullerton, CA) HPLC instrument equipped with Alltech Econosil C_{18} columns (Deerfield, IL). The NMR spectra were acquired on Varian Unity 300 or 500 spectrometers. Flash chromatography was performed on Lagand Chemicals silica gel (230-400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm) (Macherey-Nagel Inc., Easton, PA).

The ultraviolet–visible spectra were recorded with a Beckman DU-650 spectrophotometer. The pH measurements were made with a Corning pH Meter 240. Cell disruption was achieved with a Fisher 550 Sonic Dismembrator. Centrifugation was performed using either an Avanti J-25 or a J-E instrument of Beckman Coulter. Microcentrifugations were conducted with an Eppendorf 5415C microcentrifuge. Scintillation counting was carried out with a Beckman LS6500 counter.

SDS-PAGE. Protein purity and estimated molecular mass were obtained by SDS-PAGE at 25 mA per gel using a discontinuous buffer system.[23] The

separating gel and stacking gel were 12% and 4% polyacrylamide, respectively. Prior to electrophoresis, protein samples were heated for 5 min at 100 ^{o}C (or 10 min at 37 ^{o}C for samples containing high imidazole concentrations) in 62.5 mM Tris-HCl buffer (pH 6.8) containing 10% glycerol, 2% SDS, 5% β-mercaptoethanol, and 0.0025% bromophenol blue. Electrophoresis of the denatured samples was run in 25 mM Tris-HCl, 192 mM glycine, and 0.1% SDS (pH 8.3). Gels were stained with Coomassie blue (2.5 g/L of Coomassie Brilliant Blue G-250 in acetic acid : water : methanol, 1:4:5 by volume) and de-stained in ethanol : acetic acid : water (4:5:41 by volume).[24] Protein concentrations were determined according to Bradford[25] using bovine serum albumin (BSA) as the standard and dye reagent from BioRad (Hercules, CA).

Western Blot Analysis. Western blot was used to detect poly-histidine tag on recombinant proteins.[26] SDS-PAGE of the proteins of interest, containing one lane of pre-stained protein marker, was carried out as described above up to the staining step. The proteins on the gel were transblotted to the nitrocellulose membrane at 100 V for 1 h as described in the BioRad Guide using Mini Trans-Blot cell, with the running buffer containing 25 mM Tris base, 192 mM glycine, 20% v/v methanol, pH 8.3. The detection of the polyhistidine-tagged proteins was done as follows unless noted otherwise. Membrane was immersed in the blocking solution of 1% non-fat dry milk in TBST (20 mM Tris-HCl, 150 mM NaCl, 0.05% v/v Tween® 20, pH 7.5) and agitated at room temperature for 1 h. Membrane was then agitated at room temperature with the primary antibody solution (1:3,000 dilution of monoclonal anti-polyhistidine clone His-1, Sigma

H1029, in TBST) for 1 h, rinsed with TBST for 5 min three times, then incubated with the secondary antibody solution (1:30,000 dilution of anti-mouse IgG [Fc specific] alkaline phosphatase conjugate, Sigma A2429, in TBST), and rinsed again with TBST for 5 min three times. Detection was achieved by incubation of membrane in a solution of 0.33 mg/mL NBT and 0.165 mg/mL BCIP in 10 mL of an alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) in the dark for 10 to 30 min.

4.2.2 Preparation of the DesVII Substrates and Product

*TDP-D-desosamine (**22**).* The donor substrate for DesVII studies, TDP-D-desosamine (**22**), was prepared by a former group member, Dr. Cheng-wei Chang, and a visiting researcher, Dr. Haruko Takahashi, *via* hydrolysis of erythromycin to release desosamine followed by chemical conversions to its TDP form according to a published procedure.[27, 28]

Alternative Donor Substrates. TDP-D-mycaminose (**25**) and TDP-α-L-rhamnose (**108**) were obtained through chemical syntheses by former group members Dr. Huawei Chen and Dr. Alexander Wong, respectively. TDP-β-L-rhamnose (**109**) was obtained through an enzymatic synthesis by a current group member Dr. Hua Zhang.

*10-Deoxymethynolide (**65**).* Gram quantities of the acceptor, 10-deoxymethynolide (**65**), were obtained from the fermentation broth of *S. venezuelae* KdesI-80 mutant by extracting the products from the supernatant followed by silica gel purification as described in Chapter 2.[29] Typically, 6 L of KdesI-80 culture yielded approximately 1.5 g of crude oil, from which

approximately 0.7 g of pure 10-deoxymethynolide was obtained. The identity of the compound was confirmed by 300 MHz ^{1}H NMR ($CDCl_3$).

Alternative Acceptor Substrates. Narbonolide (**64**) was provided by Dr. Takahashi and was obtained from culturing various *S. venezuelae* mutants with defective sugar genes under the 14-membered ring production conditions.[30] Tylactone (**47**) and 15-methyl-6-deoxyerythronolide B (**107**) were kindly donated by Eli Lilly and Company and Kosan Biosciences, respectively.

*Preparation of DesVII Product, YC-17 (**3**).*

Fermentation of *S. venezuelae* AX906. *S. venezuelae* AX906 was provided by Professor David Sherman as a frozen spore suspension. Strain propagation and fermentation were carried out as previously reported.[31] No methymycin- or pikromycin-related macrolide products were detected in the fermentation broth despite multiple attempts, including isolation and fermentation of individual colonies.

Inhibition of monooxygenases in *S. venezuelae*. To accumulate non-hydroxylated methymycin or neomethymycin, YC-17 (**3**), wild type *S. venezuelae* was fermented in the presence of a known P450 inhibitor, xanthotoxin (or 8-methoxypsoralen), as follows. Strain was inoculated as a spore suspension (10 μL) in 5 mL of a seed medium (Chapter 2) and grown at 29 ^{o}C for 48 h. Vegetative medium was then inoculated with 100 μL of the seed culture and 0.5 M stock of xanthotoxin in DMSO was added to the growth medium to a final concentration of 2 mM. The control culture did not contain xanthotoxin. The cultures were then fermented at 29 ^{o}C for another 48 h, after which the cells and

media particles were removed by centrifugation, pH of the supernatant was adjusted to 9.5, and the products were extracted with an equal volume of chloroform. After evaporation of the solvent, the remaining residue was re-dissolved in 200 μL of chloroform, and 10 μL of each of the resulting samples were analyzed by TLC using a chloroform : methanol : 25% NH_4OH (90:9.9:0.1) solvent system. TLC plates were developed with a vanillin stain (0.75% vanilla, 1.5% v/v H_2SO_4, methanol).

Construction of KpikC *S. venezuelae* mutant. The construction of *pikC* mutant of *S. venezuelae* analogous to AX906 was undertaken. First, two fragments flanking *pikC* gene in the genome of *S. venezuelae* (pikC-A and pikC-B) and kanamycin resistance gene (*neo*) were cloned into the vector pUC119. Fragments pikC-A (924 bp) and pikC-B (881 bp) were amplified by PCR using *Hind*III-digested *S. venezuelae* genomic DNA as the template with the following primers: pikC-K12 (5′-GGCCGAATTCGTCGAAGTACAG-3′, *Eco*RI restriction site is underlined) and pikC-K2 (5′-GCGCTCTAGAGAAGCG-GTCGTTC-3′, *Xba*I) for pikC-A; pikC-K3 (5′-GGCCCTGCAGTGTGGTA-TCCGAAC-3′, *Pst*I) and pikC-K4 (5′-GCGCAAGCTTCGGGGCCGTAGT-AGTG-3′, *Hind*III) for pikC-B. The resulting PCR fragments were digested with the corresponding pairs of restriction endonucleases and purified from agarose gel using the QIAquick kit. Fragment *neo*/*Xba*I, *Pst*I was previously obtained as described in Chapter 2. Fragments pikC-A/*Eco*RI, *Xba*I , pikC-B/*Pst*I, *Hind*III, and *neo*/*Xba*I, *Pst*I were ligated into *Eco*RI and *Hind*III sites of pUC119, the

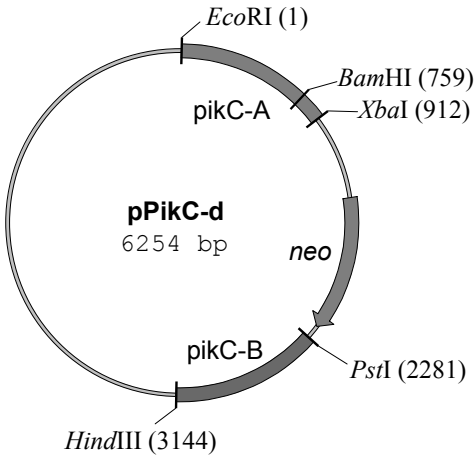

Figure 4-3: Map of the plasmid pPikC-d used for partial deletion/ replacement of *pikC* in *S. venezuelae*.

resulting construct, pPikC-d (Figure 4-3), was confirmed by a series of restriction digestions and the sequencing of the incorporated inserts.

The *Eco*RI-*Hind*III fragment (3143 bp) of pPikC-d was excised and ligated into the same restriction sites of the pKC1139 vector. The resulting construct, pPikC-K, was used to transform *E. coli* S17-1 competent cells. The homologous recombination and the screening of the double-crossover mutants were preformed as described in Chapter 2. Eight positive (Kan^R, Apr^S) clones were fermented as described and found to produce practically identical sets of metabolites with the major product being YC-17 (**3**). One of them, KpikC-2, was chosen for further experiments.

Purification of YC-17. *S. venezuelae* KpikC-2 was fermented under the 12-member ring production conditions (460 mg of crude extract per 4 L culture), and after the separation of the crude extract on silica gel column (loaded with $CHCl_3$, eluted with 0-10% gradient of methanol in chloroform containing 0.1%

v/v of 25% NH_4OH), approximately 100 mg of pure YC-17 and 200 mg of YC-17 mixed with 20% of narbomycin were obtained. The structure of YC-17 was confirmed by 300 MHz ^1H NMR (in $CDCl_3$).[32]

4.2.3 Purification of DesVII-C-His Protein

Plasmid pHC29, containing the *desVII* gene insert from *S. venezuelae* ligated into the *Nde*I and *Xho*I restriction sites of pET24b(+) vector, was prepared by Dr. Huawei Chen. The purification protocol was designed according to the QIA*expressionist*™ instruction manual (Qiagen) for purification of His-tagged proteins with Ni-NTA agarose resin. All steps were carried out at 4 °C, and the collected fractions were analyzed by SDS-PAGE.

Cell Growth. An overnight culture of *E. coli* BL21(DE3)/pHC29 was used to inoculate six 1 L portions (1–2 to 100 dilution) of LB medium containing kanamycin (25 μg/mL). The cultures were incubated at 37 °C until the OD_{600} reached 0.6–0.7 (the temperature was gradually lowered to 16 °C before the culture reached the desired OD_{600} reading). The cultures were then induced with 0.5 mM IPTG and further incubated at 16 °C for 16–18 h. The cells were harvested by centrifugation (5,000 *g*, 10 min) and used immediately in the next step.

Crude Extract. Cell pellet (typically 28 g) was re-suspended in 90 mL of lysis buffer (50 mM sodium phosphate, 300 mM NaCl, 10% glycerol, 5 mM imidazole, pH 8.0) containing 0.5 mM PMSF. Lysozyme was added to the concentration of 1 mg/mL, and the resulting suspension was incubated on ice for 30 min. The cells were disrupted by sonication in 10 sec burst for 36 times with a

20 sec cooling period between each blast. Cell debris was removed by centrifugation (16,000 *g*, 4 °C, 30 min). The resulting supernatant was used in the next step, and a sample of the pellet was kept for SDS-PAGE analysis.

Purification Using Ni-NTA Agarose. To the crude extract, 10 mL (bed volume of resin) of Ni-NTA resin pre-washed with lysis buffer was added, and the mixture was incubated with a slow agitation at 4 °C for 3 h. The suspension was loaded into the column and the flow-through fraction was collected. The resin was sequentially washed with 50 mL lysis buffer containing 5 mM imidazole, 50 mL lysis buffer containing 15 mM imidazole, and 100 mL lysis buffer containing 30 mM imidazole. The bound protein was eluted with an elution buffer containing 250 mM imidazole (except for imidazole concentration, composition is the same as in the lysis buffer). The desired fractions, as detected by SDS-PAGE, were pooled and dialyzed against three changes of 1 L of 50 mM

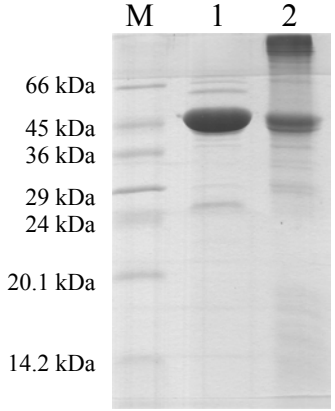

Figure 4-4: SDS-PAGE of purified DesVII-C-His (lane 1, major band of approximately 47 kDa) and partially purified DesVIII-2 (lane 2, described in Section 4.2.18). Lane M is the molecular weight marker: bovine serum albumin, 66 kDa; chicken egg ovalbumin, 45 kDa; rabbit glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; bovine carbonic anhydrase, 29 kDa, bovine trypsinogen, 24 kDa; soybean trypsin inhibitor, 20 kDa; bovine α-lactalbumin, 14.2 kDa.

Tris-HCl buffer (pH 8) containing 10% glycerol. The purified protein was concentrated (precipitated particles removed by centrifugation), aliquotted into 0.5 mL portions, flash frozen with liquid nitrogen, and stored at –80 ^{o}C (Figure 4-4, lane 1). The presence of the desired protein was confirmed by a Western blot analysis with the primary anti-polyhistidine antibody as described above. Typical yields of purified DesVII-C-His were around 100 mg/L of culture.

4.2.4 Native Molecular Weight Determination of DesVII-C-His

The native molecular weight of DesVII-C-His was determined using FPLC equipped with Superdex 200 HR 10/30 column according to a procedure recommended by the column manufacturer, Amersham Biosciences. Standards and samples were isocratically eluted using 50 mM sodium phosphate, pH 7, 0.15 M NaCl at 0.5 mL/min. The following protein standards (Sigma MW-GF-200) were injected into FPLC (250 μL each): cytochrome *c* (12.4 kDa, 4 mg/mL), carbonic anhydrase (29 kDa, 6 mg/mL), bovine serum albumin (BSA, 66 kDa, 10 mg/mL), alcohol dehydrogenase (150 kDa, 10 mg/mL), and β-amylase (200 kDa, 8 mg/mL). Blue dextran (2000 kDa, 250 μL of 2 mg/mL) was used to determine the void volume of the column. DesVII-C-His injected has a concentration of 4 mg/mL (250 μL).

4.2.5 Preliminary Glycosyltransferase Activity Assay

The composition of the assay mixture was developed by Dr. Lishan Zhao including: 50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM TDP-D-desosamine (**22**), 0.5 mM 10-deoxymethynolide (**65**, from 20 mM stock in ethanol), and 20% v/v protein component (DesVII-C-His or DesVII-C-His and various protein

crude extracts) in a total volume of 100 μL. The mixture was gently vortexed and incubated at 29 ^{o}C with shaking for about 2 h. The products and the remaining acceptor substrate were extracted from the incubation mixture with 100 μL chloroform, and the organic extract was spotted on a TLC plate (10–20 μL per spot). TLC was performed as described in the Section 4.2.2.

4.2.6 Preparation of Protein Crude Extracts from *S. venezuelae* and *S. venezuelae* KdesVIII-92 Mutant

Cell Growth. The medium used for growing *S. venezuelae* KdesVIII-92 mutant contained 50 μg/mL of kanamycin. Spore suspensions of each strain (100 μL) were inoculated into 50 mL of a supplemented minimal medium[33] with some modifications[22] as described below. Each of the solutions, with the exception of trace element solution and $(NH_4)_2SO_4$ (which were filter sterilized), was separately autoclaved, then the solutions were mixed in the following order: deionized H_2O (31.4 mL), PEG6000 (10% w/v, 50 mL), $MgSO_4$•7 H_2O (24 g/L, 2.5 mL), TES buffer (0.25 M, pH 7.2, 10 mL), a mixture of NaH_2PO_4 and K_2HPO_4 (50 mM each, 1 mL total volume), glucose (50% w/v, 2 mL), Antifoam 289 (1 mL), trace element solution ($ZnSO_4$•7 H_2O, $FeSO_4$•7 H_2O, $MnCl_2$•4 H_2O, $CaCl_2$•2 H_2O, NaCl, 100 mg/L each, 0.1 mL total volume), casamino acids (20% w/v, 1 mL), and $(NH_4)_2SO_4$ (1.5 M, 1 mL). Cultures were grown with vigorous shaking (350 rpm) at 29 ^{o}C for approximately 70 h. Proteins were isolated according to the literature procedure[34] with some modifications.

Protein Isolation and Ammonium Sulfate Precipitation. Cells were harvested by centrifugation (27,000 *g*, 4 ^{o}C, 10 min) and pellet was washed with 30 mL of 0.5 M NaCl, 50 mM potassium phosphate (pH 7.2), then 15 mL of

50 mM potassium phosphate (pH 7.2). The cells were resuspended in 5 mL of a disruption buffer (50 mM potassium phosphate, pH 7.2, 10% v/v glycerol, 2 mM DTT) and disrupted by sonication (12 cycles of 20 sec burst with a 10 sec cooling interval between bursts). The lysate was cleared by centrifugation (27,000 *g*, 4 ^{o}C, 20 min) resulting in the recovery of 4.5 mL of supernatant. Ammonium sulfate (1.6 g) was added slowly to the supernatant while stirring on ice. The protein precipitate was collected by centrifugation (as above) to give the 0–60% ammonium sulfate fraction. To the remaining supernatant 1.1 g of ammonium sulfate were added. The solution was adjusted to pH 4 with acetic acid and stirred for another 70 min. Centrifugation of this solution as above resulted in the 60–90% ammonium sulfate fraction. Pellets were resuspended in 0.75 mL of the disruption buffer and dialyzed against the same buffer. The resulting viscous yellowish solutions were frozen in liquid nitrogen, stored at –80 ^{o}C, and later used in the glycosyltransferase assays as above.

4.2.7 Expression of *desVII* in *S. lividans*: 1st Attempt

Preparation of pIJ702 DNA. Strain *S. lividans*/pIJ702 (ATCC 35287) was purchased from ATCC as freeze-dried pellet and used for the plasmid isolation. Plasmid DNA isolation from *S. lividans* proved to be difficult, but a small quantity of pIJ702 was eventually obtained through alkaline lysis and potassium acetate precipitation based on a modified literature protocol.[22]

Specifically, tryptic soy broth (TSB) medium (50 mL) was inoculated with the frozen culture of *S. lividans*/pIJ702 (0.5 mL) and incubated with vigorous shaking at 29 ^{o}C for approximately 72 h. Cells were harvested by centrifugation,

and the pellet was washed with 10% sucrose and resuspended in 3.2 mL of TEGLR (25 mM Tris-HCl pH 8, 10 mM EDTA pH 8, 50 mM glucose, 1 mg/mL lysozyme, 5 μg/mL pre-boiled RNase A). After incubation at 37 ^{o}C for approximately 30 min, 6.4 mL of 0.2 N NaOH, 1% SDS was added. The suspension was mixed immediately by several inversions and incubated at 20 ^{o}C for 20 min. To the resulting lysate the following reagents were added: 4.8 mL of 3 M potassium acetate (pH 4.8) and 0.48 mL of acid phenol/chloroform (5 g phenol, 5 mL chloroform, 1 mL H_2O, 5 mg 8-hydroxy quinoline). The lysate was mixed immediately by inversion, incubated on ice for 5 min, and centrifuged for 15 min at 12,000 *g*, 4 ^{o}C. The aqueous phase was transferred to a fresh tube; DNA was precipitated with 8.64 mL of isopropanol and recovered by centrifugation (15 min, 12,000 *g*, 4 ^{o}C). DNA pellet was washed with 6 mL of 70% ethanol, redissolved in 3.2 mL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8), and extracted four times with 3.2 mL of phenol : chloroform : isoamyl alcohol (25 : 24: 1, pH 6.5) and once with 3.2 mL of chloroform. To the aqueous layer (1.6 mL) the following reagents were added: 0.16 mL of sodium acetate (3 M, pH 5.2) and 3.52 mL of ethanol. DNA pellet was recovered by centrifugation as above, washed with 2 mL of 70% ethanol, and redissolved in 0.6 mL of TE buffer at 4 ^{o}C overnight. The resulting viscous solution contained pIJ702 plasmid DNA (as confirmed by restriction enzyme digestion and agarose gel electrophoresis) contaminated with small quantities of genomic DNA. It was used in later experiments as the source of pIJ702.

Cloning desVII in pIJ702. The fragment containing *desVII*, des7-Sl-C-His, was designed to incorporate histidine tag at the *C*-terminus of the produced DesVII. It was prepared by PCR using cosmid pLZ4 as the template and the following primers: des7-Sl-forward (5′-GGCCGCATGCGCGTCCTGCTGAC-3′) and des7-Sl-reverse-His (5′-GCGCAGATCTCA**GTGGTGGTGGTGGTG-GTG**CCGGGCGTC-3′). Underlined are the *Sph*I and *Bgl*II restriction sites, respectively; nucleotides encoding the six histidine residues are shown in bold. This fragment was ligated into the *Sph*I-*Bgl*II restriction sites of pIJ702, and the resulting construct was used to transform protoplasts of *S. lividans* 1326.

Published procedures were followed for the preparation of *S. lividans* 1326 protoplasts[22] transformation and selection.[21] For transformation, fresh PEG 1000 and three different ratios of T buffer to PEG (2:1, recommended 3:1, and 4:1) were used. Strain *S. lividans* 1326 transformed with pIJ702 was a positive control, and the same strain with no DNA added during transformation was a negative control. Transformants were plated onto R2YE plates,[22] incubated at 30 ^{o}C for 20 h, and then overlaid with a thiostrepton solution (200 μg/mL of agar).

Selection of the S. lividans/desVII-Sl-C-His/pIJ702 Clones. Colonies became visible after several (5 to 7) days at 30 ^{o}C, from which 20 colonies were picked and streaked on R2YE agar containing thiostrepton (20 μg/mL) in slanted tubes. The tubes were incubated at 30 ^{o}C for several days, and a piece of the agar was used to inoculate 5 mL portions of TSB media containing thiostrepton (10 μg/mL). The resulting culture was grown for several days. Several attempts

were made to isolate DNA of desVII/pIJ702 plasmids from the above *S. lividans*/desVII/pIJ702 cultures for analysis. Unfortunately, no or very low yields of DNA were produced since no or little desired bands were visible on agarose gel after the digestion.

To check for protein production, fresh cultures of transformants were grown as above, and cell pellets were collected by centrifugation followed by re-suspension in a sonication buffer #2 (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM DTT).[35] After sonication (three pulses, 20 sec each), proteins were separated by SDS-PAGE and blotted onto the HybondC Extra membrane. Detection using anti-His primary antibody, alkaline phosphatase conjugate and NBT/BCIP substrates failed to identify a visible band for any of the 20 clones.

4.2.8 Expression of *desVII* in *S. lividans*: 2nd Attempt

Plasmid pSB01was designed for the expression of *desVII* in *S. lividans*. Unlike desVII/pIJ702, it has the *desVII* gene cloned downstream to *melC1* of pIJ702 as opposed to replacing it. Vector pUC119 was used as the base for the construct to enable cloning in *E. coli* rather than *Streptomyces*. Kanamycin resistance marker, *neo*, was also introduced to improve selection of transformants in *S. lividans*.

Preparation of Plasmid pSB01. The design of the expression plasmid is shown in Figure 4-5 (Route A). The **melC1** fragment used contains not only the *melC1* sequence but also the region between *melC1* and *melC2* present in the original *S. antibioticus* sequence. The **desVII** fragment contains the sequence coding for the *C*-terminal His_6 tag which was introduced as part of the PCR

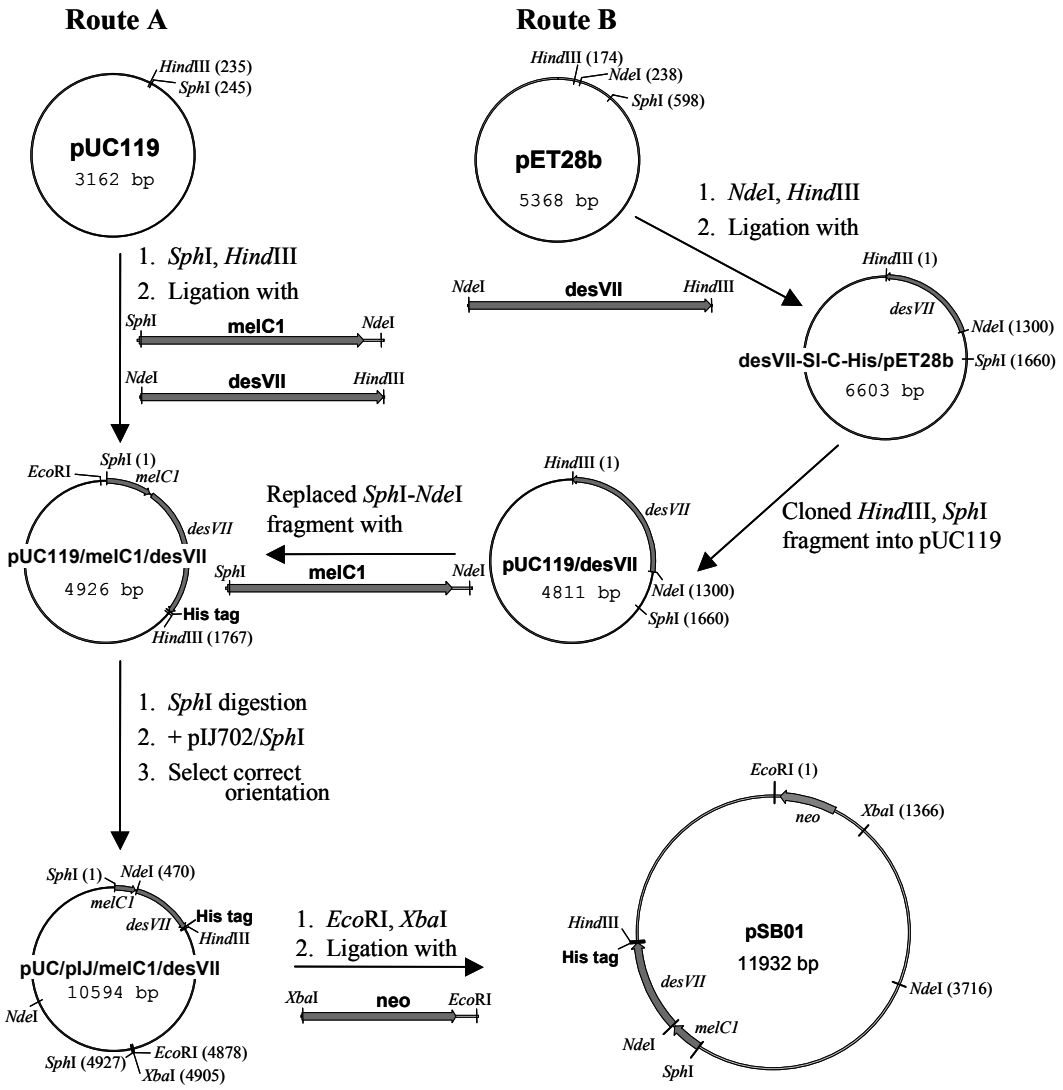

Figure 4-5: Strategy for constructing pIJ702/melC1/desVII plasmid for *desVII* expression in *S. lividans*.

primer. Vector pIJ702 contains thiostrepton resistance gene. All cloning steps were done in *E. coli* DH5α, and ampicillin was used as a selection marker at the concentration of 100 μg/mL.

Fragment **melC1** was amplified by PCR using melC1-forward (5′-GCGCG<u>GC**ATGC**</u>CGGAACTCAC-3′, *Sph*I site is underlined, start codon of *melC1* is shown in bold) and melC1-reverse (5′-GCGCG<u>CAT**ATG**</u>CGGG-TGCTC-3′, *Nde*I site is underlined, start codon of *melC2* is shown in bold) as primers, and vector pIJ702 as the template. Fragment **desVII** was amplified by PCR using des7-Sl-up2 (5′-GGCC<u>CAT**ATG**</u>CGCGTCCTGCTGAC-3′, *Nde*I site is underlined, start codon of *desVII* is shown in bold) and des7-Sl-C-His2 (5′-GCGC<u>AAGCT**TCA**</u>*GTGGTGGTGGTGGTGGTG*CCGGGCGTC-3′, *Hind*III site is underlined, residues encoding histidine tag are italicized, stop codon is shown in bold) as primers and cosmid pLZ4 as the template. The ends of these fragments were freed by a digestion with an appropriate pair of restriction endonucleases. The three-way ligation of **melC1**/*Sph*I, *Nde*I and **desVII**/*Nde*I, *Hind*III in the *Sph*I-*Hind*III restriction sites of pUC119 was not successful. Therefore, an alternative path (Route B, Figure 4-5) was undertaken.

To avoid the three-way ligation step (it has lower efficiency than the two-way ligation and, without a marker, it is hard to select for a three-way ligation product) **desVII**/*Nde*I, *Hind*III fragment was first cloned into the pET28b vector. The 1.66 kb *Hind*III-*Sph*I fragment of the desVII-Sl-C-His/pET28b plasmid was then cloned into pUC119, and the *Sph*I-*Nde*I fragment of the resulting pUC119/desVII was replaced with **melC1**/*Sph*I, *Nde*I to generate the desired pUC119/melC1/desVII plasmid, which is also the anticipated product of the three-way ligation (Figure 4-5).

Plasmid pUC119/melC1/desVII and vector pIJ702 were both digested with *Sph*I and subjected to the T4 DNA ligase catalyzed reaction. Ampicillin-resistant clones resulting from the ligation between pUC119/melC1/desVII and pIJ702 were observed. The desired clone with the correct orientation of pIJ702 relative to pUC119/melC1/desVII was selected based on its *Bam*HI digestion pattern. The identity of the clone pUC/pIJ/melC1/desVII was confirmed by DNA sequencing.

The fragment containing kanamycin resistance gene, **neo**/*Xba*I, *Eco*RI was then cloned into the pUC/pIJ/melC1/desVII plasmid. Fragment **neo** was amplified by PCR from plasmid pDes1-d1 (Chapter 2) using the following primers: 1neomycin (5′-CGCGTCTAGATACCTACAGCGTGAGC-3′, *Xba*I site is underlined) and 2neo2 (5′-CGCGGAATTCCCACGAATTAGCC-3′, *EcoR*I site is underlined). The resulting plasmid, pSB01, confers resistance to ampicillin in *E. coli* and to kanamycin and thiostrepton in *Streptomyces* strains. pSB01 was used in later experiments.

Expression of desVII in S. lividans TK64. Plasmid pSB01 was used to transform the protoplasts of *S. lividans* TK64 (ATCC 69441) as described above. Transformants were selected by overlaying R2YE plates containing transformed protoplasts with soft nutrient agar[22] supplemented with kanamycin to bring the final concentration of antibiotic in the agar to 50 μg/mL. Thirteen positive colonies (Kan^R) were propagated on R2YE plates containing kanamycin (50 μg/mL) and thiostrepton (5 μg/mL). The clones were inoculated in 5 mL of a modified GYM medium (per 1 L: 4.0 g glucose, 4.0 g yeast extract, 10.0 g malt

extract, pH 7.2)[36, 37] containing kanamycin (50 μg/mL) and thiostrepton (5 μg/mL) and grown at 29 ^{o}C for 72 h.

Aliquots of GYM cultures (0.5 mL) were taken and DNA was isolated using QIAprep kit from QIAgen (200 μg/mL of lysozyme was added to the P1 resuspension buffer). The isolated DNA was subjected to PCR analysis using des7-Sl-up2 and des7-Sl-C-His2 primers. The reactions of five of the clones produced the expected 1.3 kb fragments, therefore confirming the presence of the pSB01 plasmid.

The cultures of the four positive clones were harvested by centrifugation, cell pellet was resuspended in SDS-PAGE sample buffer, and supernatant was concentrated 5-fold using Microcon YM-30. Both pellet and supernatant were analyzed using SDS-PAGE. All of the supernatant fractions contained a band at approximately 47 kDa (expected size of His_6-tagged DesVII is 47.2 kDa) indicating the presence of DesVII-C-His. There was no such band visible from the pellet fractions.

Concentrated supernatant fractions were used as the protein component in the glycosyltransferase assay (as described above) using 10-deoxymethynolide (**65**) and TDP-D-desosamine (**22**) as substrates in the presence of and Mg^{2+} and Mn^{2+} metal ions. The extent of the reaction was evaluated by TLC analysis.

4.2.9 Purification of Sfp Protein

The expression plasmid containing *Bacillus subtilis sfp* gene, pET29-Sfp, was kindly provided by Dr. Christopher Walsh, Harvard Medical School. It consists of a 0.7 kb *sfp* gene (Accession Number X63158)[38] cloned into the *Nde*I-

*Xho*I sites of the expression vector pET29b(+). It allows the production of Sfp fused with a *C*-terminal His_6 tag.

Cell Growth. The expression plasmid pET29-Sfp was used to transform *E. coli* BL21, and the resulting strain was grown at 37 °C overnight in LB medium supplemented with kanamycin (50 μg/mL). The overnight culture was diluted 100 fold in 3 L of LB medium (35 μg/mL of kanamycin) and incubated at 37 °C until OD_{600} reached 0.4. The production of Sfp was induced with 0.16 mM IPTG, and the cells were incubated at 18 °C for 16 h. The cells were harvested by centrifugation (5,000 *g*, 10 min) and used immediately in the next step.

Sfp Purification. Crude protein extract (from 20 g of cell pellet) preparation and purification of Sfp by Ni-NTA affinity chromatography were the same as described for the purification of DesVII-C-His, with the only exception being that no protease inhibitor, PMSF, was used this time. The desired protein fractions, as detected by SDS-PAGE, were pooled and dialyzed against 40 mM potassium phosphate buffer (pH 7.2) containing 20% glycerol. The identity of the protein was confirmed by the Western blot analysis with an anti-histidine detection system. The purified protein was concentrated, aliquotted, flash frozen with liquid nitrogen, and stored at –80 °C. Protein was estimated to be >95% pure (SDS-PAGE), and its concentration was determined to be 45 mg/mL by the Bradford assay. The yield of the purified Sfp was 75 mg/L of culture.

4.2.10 Purification of apo-ACP_5

Construction of E. coli Strain Producing ACP_5 domain of PikAIII PKS of S. venezuelae. The DNA fragment containing the region coding for ACP_5 was

amplified by PCR from *Bam*HI-digested *S. venezuelae* genomic DNA using forward primer 5′-GCCGCATATGACGGTGTCCCGTCCCAGCCTTC-3′ and reverse primer 5′-GCGCAAGCTTGGTGTTACGGGGGCCGAGAGCCAT-CCGGATCAG-3′ (restriction sites *Nde*I and *Hind*III, respectively, are underlined). This fragment was cloned into the *Nde*I-*Hind*III sites of pET24b(+). The resulting plasmid, ACP5-2/pET24b, was used to transform *E. coli* BL21.

Purification of ACP_5-C-His. Strain *E. coli* BL21/ACP5-2/pET24b was grown overnight in LB medium (kanamycin, 50 μg/mL). The overnight culture was used to inoculate 6 L of LB medium (kanamycin, 35 μg/mL), grown at 37 ^{o}C until OD_{600} reached 0.75, and the production of ACP_5-C-His was then induced with 1 mM IPTG. After incubation at 37 ^{o}C for an additional 4 h, cells were harvested by centrifugation and ACP_5-C-His was purified using Ni-NTA agarose as described above for DesVII-C-His purification with the following modifications. Lysis buffer was 20 mM Tris-HCl (pH 8), 0.2 M NaCl, and 1.5 mM imidazole. After the incubation with the protein crude extract, Ni-NTA resin was extensively washed with the lysis buffer. The weakly bound proteins were then washed out with a lysis buffer containing 20 mM imidazole. Finally, bound ACP_5-C-His was eluted using a lysis buffer containing 300 mM imidazole. The fractions containing the desired protein (23.6 kDa) were combined and dialyzed against 50 mM Tris-HCl (pH 8) buffer containing 10% glycerol (Figure 4-6, lane 1).

ACP_5-C-His was further purified by anion exchange chromatography using FPLC equipped with a MonoQ HR 10/10 column. Proteins were loaded on

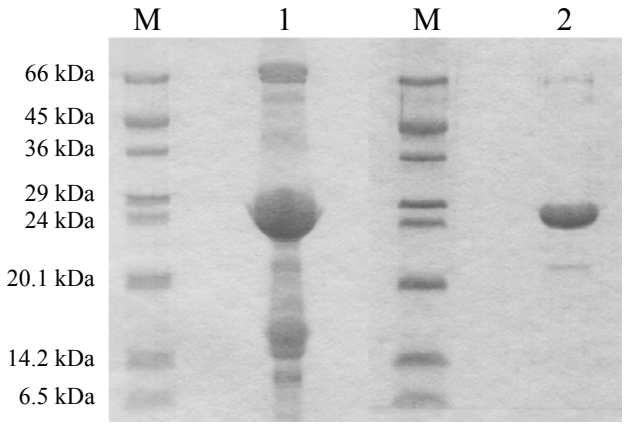

Figure 4-6: SDS-PAGE illustrating purification of ACP_5. Lane 1: protein sample after Ni-NTA column, lane 2: ACP_5 after MonoQ purification. Lane M is the molecular weight marker and bands are as in Figure 4-4 with an additional band of bovine aprotinin (6.5 kDa).

the column with buffer A (50 mM Tris-HCl, pH 8, 10% glycerol) at 3 mL/min over 4 column volumes and then eluted with the gradient 0–40% of buffer B (buffer A plus 1 M KCl) in buffer A over 20 column volumes. Fractions containing ACP_5-C-His (at approximately 220 mM KCl) were pooled and desalted using Centriprep YM-10 until approximately 40 mM KCl remained. They were flash frozen with liquid nitrogen and stored at –80 ^{o}C (Figure 4-6, lane 2). Protein concentration was 15 mg/mL, and the overall yield of pure ACP_5-C-His was 10 mg/L of culture.

4.2.11 Model Reaction for Acyl-phosphopantetheinylation of apo-ACP_5

General. Assays were carried out according to the published procedure with minor modifications.[39] Radiolabeled acetyl coenzyme A [acetyl-^{3}H] from ICN Biochemicals was received as 0.1 mCi/mL solution in 0.01 M sodium acetate

(pH 5) and stored at –20 ºC. It exhibits approximately 1–2% decomposition per month under these conditions.

Radioassay Procedure. A typical assay of 100 μL contained 50 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 5 mM DTT, and 40 mM NaCl. To this mixture were added 3 μL of 0.64 mM apo-ACP_5-C-His (in 50 mM Tris-HCl, pH 8, 10% glycerol, final concentration 19.2 μM), 5 μL of 35.34 μM (1.77 μM, 2.83×10^3 μCi/μmol) acetyl [^{3}H]-CoA solution, and 1 μL of 20 μM Sfp (in 50 mM potassium phosphate, pH 7.5, 10% glycerol, final concentration 0.2 μM). Reaction mixtures were vortexed before and immediately after the addition of Sfp and incubated at room temperature for periods of 0.5, 1, 1.5, 2, 4 and 6 min. Two control samples, one without Sfp and one without ACP, were prepared and incubated for 2 min. The reactions were quenched by adding 800 μL of 10% TCA; then 50 μL of 10 mg/mL BSA solution was added as a carrier. Samples were centrifuged, and the pellet was washed twice with 10% TCA. Pellets were resuspended in 100 μL of formic acid, a scintillation liquid was added, and the samples were counted. Readings of disintegrations per minute (DPM) were converted to the unit of mole product by the equality, 2.22×10^6 DPM = 1 μCi radioisotope and the specific activity of the compound used (μCi/μmol).

4.2.12 Purification of TE-1 and TE-2 Proteins

Construction of Expression Plasmids pTE-C-His and pTE(2)-C-His. The fragments to be inserted in the expression vector, te-1 and te-2, were amplified by PCR from the *S. venezuelae* genomic DNA digested with *Hind*III. Primers TE-b1 (5′-GCCGCATATGTTCCGCGCCCTGTTCCGG-3′) and TE-b2 (5′-GCGC-

AAGCTTCTTGCCCGCCCCCTCGAT-3′) were used to amplify the te-1 fragment (restriction sites *Nde*I and *Hind*III, respectively, are underlined); and primers TE-b3 (5′-GCCGCATATGTCCGGGGCCGACACCGGCG-3′, restriction site *Nde*I is underlined) and TE-b2 – for the te-2 fragment. After digestion with *Nde*I and *Hind*III, te-1 and te-2 were separately cloned into the same restriction sites of the pET24b(+) vector. The resulting plasmids, pTE-C-His and pTE(2)-C-His, were used to transform the competent cells of *E. coli* BL21.

Affinity Purification of TE-1 and TE-2. The overnight cultures of strains *E. coli* BL21/TE-1 and *E. coli* BL21/TE-2 were inoculated into 1 L of LB medium containing 35 μg/mL of kanamycin (1 to 100 dilution) and grown at 37 ^{o}C until OD_{600} reached 0.6. The production of TE was then induced by the addition of IPTG to a final concentration of 50 μM, and cells were grown at 16 ^{o}C for an additional 17 h. The preparation of the crude protein extract and the purification of TE by nickel affinity chromatography followed the same procedures as described for DesVII-C-His purification (Section 4.2.3). Proteins eluted with a buffer containing 250 mM imidazole were dialyzed against 50 mM Tris (pH 8) containing 10% glycerol, concentrated, and stored at –80 ^{o}C.

Assay of TE Activity. The reaction mixture of 50 μL contained 50 mM Tris-HCl (pH 9) or 50 mM sodium phosphate (pH 8) and 1 mM 10-deoxymethynolide (**65**). The amount of TE used varied from 0.5 to 2 μM. The reaction mixtures were incubated at 29 ^{o}C for up to 16 h and the remaining 10-deoxymethynolide was extracted with chloroform. The success of the

transformation was judged by TLC based on the consumption of the substrate as compared to the control which lacks TE. No reaction was discernible under the assay conditions using different preparations of TE.

4.2.13 Construction and Characterization of *S. venezuelae* KTE Mutant

Generation of Disruption Plasmid pTE-K. Disruption plasmid pTE-K was generated from plasmid pDesVIII-K (Chapter 2) by the replacement of *Eco*RI, *Xba*I fragment **sug-u** with a portion of *pikAIV*, fragment **KTE**. This replacement effectively results in deletion of part of *desVIII* plus a region upstream to *desVIII*. The fragment **KTE** was amplified by PCR from the *Bam*HI-digested *S. venezuelae* genomic DNA using primers KTE-1 (5′-GGCCGAATTC-CTTCACCCACTTCATCGAG-3′, *Eco*RI restriction site is underlined) and KTE-2 (5′-GCGCTCTAGA**TCA**[GTG][GTG][GTG][GTG][GTG][GTG]GAAGGA-GGTGCTGAG-3′, *Xba*I restriction site is underlined, stop codon is in bold, and histidine codons are in square brackets). Fragment **KTE** was then digested with *Eco*RI and *Xba*I and cloned into the respective sites of pDesVIII-K.

Homologous Recombination and Screening for Double Crossover Mutants. *E. coli* S17-1 was transformed with the resulting pTE-K plasmid and used as a donor strain for the conjugal transfer to the wild type *S. venezuelae* as described in Chapter 2. After an antibiotic selection, plasmid curing and replica screening, two strains with the desired phenotype (Kan^R, Apr^S) were obtained. They were grown in 25 mL of TSB medium containing 50 μg/mL kanamycin for 48 h at 29 ^oC, and their genomic DNA was isolated using the Kirby mix.[21]

Genomic DNA of the mutants and the wild type *S. venezuelae* was digested with *Pst*I, separated by agarose gel electrophoresis, and transferred to a nylon membrane using standard procedures.[21] DIG-labeled probes were prepared using PCR DIG Probe Synthesis Kit from Roche (Palo Alto, CA). Plasmid pTE-K was employed as the template along with the following primers to amplify the target genes: KTE-1 and KTE-2 for **KTE** (see above), des8-d1 and des8-d2 for **desVIII**, and 1neomycin and 2neomycin for **neo** (see Chapter 2 for the sequences of the last four primers). Membranes were probed with DIG-labeled fragments and hybridized DNA fragments were visualized using NBT/BCIP colorimetric detection as described in Roche's "DIG User's Manual".

Analysis of Macrolide Production by KTE Mutants. Both mutant strains, KTE-1 and KTE-2, were grown in the 12-membered ring macrolide production medium, and the production of macrolides was analyzed by TLC (Chapter 2). Alternatively, SCM medium was also used for 12-membered macrolide production. The recipe per 1 L included: 15 g soluble starch, 20 g of soytone, 0.1 g $CaCl_2$, 1.5 g of yeast extract, 10.5 g of MOPS, adjusted to pH 7.2.[40] For the production of 14-membered macrolides, PGM medium was used except that $CaCl_2$ was replaced by $CaCO_3$. The ingredients per 1 L included: 10 g of glucose, 10 g of glycerol, 10 g of peptone, 5 g of beef extract, 5 g of NaCl, 2 g of $CaCO_3$, adjusted to pH 7.3.[30] For the growth of KTE mutant, the medium contained kanamycin (50 μg/mL). It was inoculated with a spore suspension of the strain for small scale cultures (0.001 dilution) or a 2-day old TSB culture for large scale growth (0.01 dilution). A culture of the wild type *S. venezuelae* was grown in

parallel as the control. The cultures were grown at 29 ^{o}C for 2-4 days and then centrifuged. Supernatant was extracted with chloroform for TLC analysis, and proteins of the cell pellet were isolated on some occasions.

Analysis of Proteins in KTE-2. The wild type *S. venezuelae* and its KTE-2 mutant strain were grown in a supplemented minimal medium (Section 4.2.6), SCM, or PGM medium (25 to 100 mL scale) as described above. Crude proteins were isolated using the procedure outlined in Section 4.2.6 and analyzed by SDS-PAGE or the Western blot hybridization. Three different detection systems were used for Western blot: (i) mouse anti-His primary antibody, anti-mouse IgG antibody which is alkaline phosphatase conjugated, NBT/BCIP colorimetric detection (procedure as above); (ii) mouse anti-His primary antibody, anti-mouse IgG antibody which is alkaline phosphatase conjugated, chemiluminescent detection (WesternBreeze® kit from Invitrogen); (iii) single antibody INDIA™HisProbe™-horseradish peroxidase (HRP) conjugate and SuperSignal® West Pico chemiluminescent substrate (SuperSignal® West HisProbe™ kit from Pierce, Rockford, IL). For the chemiluminescence detection, after incubation with the substrate solution, the membrane was exposed to the film for a period varying from 30 s to 5–10 min and then developed.

Crude extracts were also separated on Ni-NTA agarose using the procedure outlined for DesVII-C-His purification in Section 4.2.3. Fractions from Ni-NTA column were again subjected to SDS-PAGE and Western blot analyses.

Alternatively, the cultures were grown in 200 mL of TSB medium (inoculated with 0.5 mL of a 24 h-old TSB seed culture, to which kanamycin was

added for the growth of KTE-2) for 19 h, followed by protein extraction and fractionation by solubility using partial bacterial proteome extraction kit ProteoExtract™ from Calbiochem (EMD Biosciences, Inc, San Diego, CA). The fractions were analyzed by SDS-PAGE and WesternBreeze® Western blot.

4.2.14 Preparation and Macrolide Production Analysis of desVIII/KTE, te/KTE, and te+desVIII/KTE *S. venezuelae* Strains

S. venezuelae desVIII/KTE. Plasmid pDesVIII (Chapter 2) was used to complement *S. venezuelae* KTE-2 and to generate *S. venezuelae* desVIII/KTE mutant (Kan^R, Apr^R) using the procedures described in Section 2.2.9.

Construction of Expression Plasmid TE-a/pAX702. The fragment of *S. venezuelae* DNA containing the TE domain was cloned into the *Eco*RI-*Nsi*I sites of the *Streptomyces* expression vector pDHS702. Vector pDHS702 contains *pikAI* promoter and confers resistance to thiostrepton.[41] Fragment TE-a was amplified by PCR from the *S. venezuelae* genomic DNA digested with *Hind*III. Forward primer TE-a1 (5′-GCCGGAATTC**TAA**GC[GGAAG]ACCACACCCAG-[ATG]TTCCGCGCCCTGTTC-3′) contains *Eco*RI restriction site (underlined), stop codon (bold), and DNA sequence preceding *pikAIV* (small caps) with the putative ribosome binding site (RBS) [GGAAG] and *pikAIV* start codon [ATG], followed by the 5′-terminal *te* sequence. Reverse primer TE-a2 (5′-GCGC-ATGCAT**TCA**CTTGCCCGCCCCCTCGAT-3′) contains the *Nsi*I restriction site and the 3′-terminal sequence of *te*, including the stop codon TGA (bold). Expression plasmid TE-a/pAX702 was used to transform *E. coli* S17-1, and the resulting strain was the donor strain for the conjugation experiments.

S. venezuelae te/KTE and te+desVIII/KTE. Plasmid TE-a/pAX702 was separately introduced into the *S. venezuelae* strains KTE-2 and desVIII/KTE through a conjugal transfer using *E. coli* S17-1 as the donor strain, which was transformed with this plasmid as previously described (Section 2.2.9). The resulting strains were propagated in a medium containing kanamycin and thiostrepton (50 μg/mL each) for te/KTE and kanamycin, apramycin and thiostrepton (50 μg/mL each) for te+desVIII/KTE.

Macrolide Production Analysis. All three strains, desVIII/KTE, te/KTE, and te+desVIII/KTE, together with the wild type control strain were each grown at 29 ^{o}C in a seed medium (5 mL) supplemented with the appropriate antibiotics for 48 h. A 0.3 mL seed culture from each 2-day culture was inoculated into 15 mL vegetative media and grown for additional 48 h. The cultures were harvested by centrifugation. The supernatant was adjusted to pH 9.5 and extracted with chloroform. The chloroform fractions were concentrated and analyzed by TLC as described before.

4.2.15 Feeding *S. venezuelae* Mutants with 10-Deoxymethynolide (65)

The antibiotics required for selective growth of the mutants were used in the growth media at concentrations of 50 μg/mL. Portions of 5 mL seed medium were inoculated with 10 μL of strains' spore suspensions, and the resulting mixture was incubated at 29 ^{o}C for 48 h. Seed cultures (2 mL) were then transferred into 100 mL of vegetative medium and grown for 31 h. Stock of 10-deoxymethynolide (**65**, 100 mM in ethanol) was added to the vegetative cultures to the final concentration of 50 μM, and the cultures were incubated for additional

48 h. Cells were removed by centrifugation, supernatant adjusted to pH 9.5, and extracted with an equal volume of chloroform. The solvent was evaporated and crude products redissolved in 2.5 mL of chloroform. Crude products (5 μL) were then analyzed by TLC as described above.

4.2.16 Simultaneous Expression of *pikAV* and *desVIII*: DesVIII-1

Construction of Expression Plasmid pikAV-desVIII/pET24b. DNA fragment containing *pikAV* and *desVIII* was amplified by PCR using cosmid pLZ4 as the template and des8pik-up1 (5′-GCCGCATATGACCGACAGAC-3′, the *Nde*I) and des8pik-down1 (5′-GCGCAAGCTTGGAGCTGCTGAC-3′, the *Hind*III restriction site is underlined) as primers. This fragment was cloned into the *Nde*I-*Hind*III restriction sites of the expression vector pET24b(+). The resulting plasmid pikAV-desVIII/pET24b was used for transformation of *E. coli* BL21.

Purification of DesVIII. The protocols used for the growth of the recombinant *E. coli* strain containing the expression plasmid pikAV-desVIII/pET24b (6 L culture), the preparation of protein crude extract, and the nickel affinity chromatography were the same as described in Section 4.2.3. Since no binding to Ni-NTA agarose was achieved, the flow-through fraction was further subjected to anion exchange chromatography on DEAE Sepharose CL-6B resin.

The sample was loaded onto a DEAE column pre-equilibrated with the loading buffer (50 mM potassium phosphate, pH 7.5, 15% glycerol) at 4 ^{o}C. Proteins were then eluted using a linear gradient between equal volumes (1 L) of

the loading buffer containing 0.2 M KCl and 0.8 M KCl. The fractions of about 15 mL were collected, and those containing proteins (as judged by UV A_{280} readings) were further analyzed by SDS-PAGE. Fractions 3–8 containing PikAV as the major component and fractions 21–26 containing DesVIII-1 were separately combined and dialyzed against the loading buffer. The pooled sample of DesVIII-1 contained about 30% DesVIII-1 (43 kDa) as judged by SDS-PAGE; the remaining impurities were mostly of higher molecular weight proteins.

4.2.17 Optimized Glycosyltransferase Activity Assay and Preparative TLC

The optimized assay composition was as follows (in the order of addition): water (enough to bring the total volume to 50 μL), 50 mM Tris-HCl (pH 9), 6 mM $MgCl_2$, 1 mg/mL BSA, 1 mM 10-deoxymethynolide (**65**) (from 50 mM stock in ethanol), 0.8 mM TDP-D-desosamine (**22**), 5 μL of DesVII-C-His (100 mg/mL, 2 mM), and 5 μL of DesVIII (undetermined concentration). When the effect of DesVIII concentration was studied, only 1-2 μL of DesVII-C-His and up to 9 μL of DesVIII were used to keep 20% v/v ratio of the added protein solution in the total assay cocktail so as to limit the amount of glycerol in the assay. The protein portion was substituted with the same amount of buffer in the control reactions. The mixture was vortexed and incubated at 29 ^{o}C with shaking from 2 to 19 h. To monitor the extent of the reaction, a 20 μL aliquot of each reaction mixture was extracted with an equal volume of chloroform, and the organic extract was spotted on TLC plate. TLC was performed as described in the Section 4.2.2.

The preparative TLC was used to isolate reaction product. The reaction was carried out in 100 μL scale overnight and extracted with 100 μL of chloroform. Extracts of all samples were applied on a Polygram Sil G/UV_{254} plate (4×8 cm, thickness 0.25 mm) and developed in the solvent system described above. Strips of the TLC plate were cut off and visualized with vanillin stain to determine the location of the desired compound. The silica coating at the same area on the remaining unstained portion of the plate was scraped off, and the compounds were eluted with three 300 μL portions of methanol. Methanol was evaporated; the residue redissolved in 30 μL of methanol and submitted to a high-resolution mass spectroscopy analysis by the chemical ionization technique in the positive ion mode. The results were as follows: calculated $[M+H]^+$ for $C_{25}H_{44}NO_6$ 454.3169, found 454.3161.

4.2.18 Purification of DesVIII-2 Under Denaturing Conditions

Cloning and Expression. Gene *desVIII* was amplified by PCR using pLZ4 cosmid as the template and des78-His-up (5′-GCCGCATATGACCGACGACC-TGAC-3′, the *Nde*I restriction site is underlined) and des8pik-down1 (Section 4.2.16) as primers. The fragment was cloned into the *Nde*I-*Hind*III restriction sites of pET24b(+), and the resulting plasmid, pDesVIII-C-His, was used to transform *E. coli* BL21 (or *E. coli* NovaBlue in a separate experiment).

The purification protocol was designed according to the QIA*expressionist*™ instruction manual (Qiagen) for the purification of histidine-tagged proteins. The overnight culture of *E. coli* BL21/pDesVIII-C-His was inoculated into 4 L of LB medium containing 25 μg/mL kanamycin (1 to 50

dilution) and grown at 37 °C until OD_{600} reached 0.5. The production of DesVIII-2 was induced by 1 mM IPTG, and incubation was continued at 37 °C for additional 5 h. Cells were harvested by centrifugation, cell pellet was resuspended in 50 mL of the lysis buffer (50 mM sodium phosphate, pH 8, 0.3 M NaCl, 10% glycerol, 5 mM imidazole, 0.4 mM PMSF) in the presence of 1 mg/mL lysozyme and incubated at 4 °C for 30 min. Cells were disrupted by sonication, and the supernatant was removed after centrifugation. Pellet was washed by the lysis buffer, and proteins were solubilized in 40 mL of Buffer B (100 mM sodium phosphate, 10 mM Tris, 8 M urea, pH 8) at room temperature for 1 h. Cell debris was removed by centrifugation, and crude proteins were subjected to the nickel affinity purification under denaturing conditions.

Under denaturing conditions, DesVIII-2 did not bind to the nickel resin; instead it was found in all fractions in equal amounts. Therefore, it was dialyzed against the lysis buffer with three changes (500 mL each), clarified by centrifugation, and incubated with Ni-NTA resin (10 mL bed volume) overnight at 4 °C. It was then subjected to a routine purification as described in Section 4.2.3. The fractions collected were dialyzed against 50 mM Tris-HCl, pH 8, 10% glycerol, and concentrated until a precipitate began to appear. A further concentration led to a significant protein precipitation: thus, only approximately 1 mg/mL DesVIII-2 sample was obtained. SDS-PAGE of DesVIII-2 (43.6 kDa) is shown in Figure 4-4 (lane 2). Western blot analysis confirmed the presence of a polyhistidine tag in the purified DesVIII.

4.2.19 Purification of DesVIII-3

Cloning and Expression. Gene *desVIII* was amplified by PCR using pLZ4 cosmid as the template and des78-His-forward (Section 4.2.18) and des8-reverse (5′-GCGCAAGCTTCAGGAGCTGCTGACCG-3′, the *Hind*III restriction site is underlined) as primers. This fragment was cloned into the *Nde*I-*Hind*III restriction sites of pET28b(+), and the resulting plasmid, pDesVIII-N-His, was used to transform *E. coli* BL21.

The overnight culture of *E. coli* BL21/pDesVIII-N-His was inoculated into 3 L of LB medium containing 25 μg/mL kanamycin (1/50 dilution) and grown at 37 ^{o}C until OD_{600} reached 0.5. The production of DesVIII-3 was induced by 0.5 mM IPTG, and incubation was continued at 16 ^{o}C for additional 21 h. Cells were harvested by centrifugation. The cell pellet was resuspended in 50 mL of a lysis buffer (50 mM sodium phosphate pH 8, 0.3 M NaCl, 10% glycerol, 5 mM imidazole, 0.4 mM PMSF) with 1 mg/mL lysozyme and incubated at 4 ^{o}C for 30 min. Cells were disrupted by sonication, and the supernatant was collected after centrifugation.

Purification Using Affinity, Ion Exchange and Size Exclusion Chromatography. The above crude extract was subjected to the nickel affinity chromatography as described in Section 4.2.3. Despite the fact that the presence of a histidine tag in DesVIII-3 was confirmed by the Western blot analysis, this fusion protein failed to bind to the resin. Therefore, in the following protein preparations the crude extract was prepared as above except that 50 mM sodium phosphate, pH 8, 1 mM DTT, 0.1 mM PMSF was used as the lysis buffer. Crude

extract was loaded onto a DEAE Sepharose CL-6B column and washed with 50 mM potassium phosphate, pH 8, 1 mM DTT, 0.1 mM PMSF. Bound proteins were then eluted using a linear gradient between equal volumes (0.5 L) of a loading buffer containing no KCl and 1 M KCl. Fractions of about 8 mL were collected throughout the elution, and the ones containing proteins (as judged by UV A_{280} readings) were further analyzed by SDS-PAGE.

The selected DEAE fractions were pooled, concentrated, and loaded onto a Sephacryl S200 HR column equilibrated with the running buffer. Proteins were isocratically eluted with 25 mM Tris-HCl, pH 7.5, 125 mM NaCl, 1 mM DTT, and fractions analyzed by UV-vis and SDS-PAGE as described above.

4.2.20 Purification of DesVIII-4 *via* Fusion with Maltose Binding Protein

Cloning and Expression. The expression vector MalE-pET was provided by Peng Gao. The *desVIII* gene was cloned into the *Nde*I-*Hind*III sites of the MalE-pET vector using the same insert fragment as for the pDesVIII-N-His plasmid. The depiction of the resulting plasmid pDesVIII-MalEpET is shown in Figure 4-7. It was used to transform the expression host *E. coli* BL21.

An overnight culture of *E. coli* BL21/pDesVIII-MalEpET was used to inoculate 6 L of LB medium containing kanamycin (25 μg/mL), and the cells were grown at 37 ^{o}C until OD_{600} reached 0.5–0.6. The temperature was then lowered to 18 ^{o}C. The production of the protein was induced with 0.3 mM IPTG for 15 h. The cells were harvested by centrifugation, and the cell pellet (35–40 g) frozen at –80 ^{o}C for 1 h to facilitate cell lysis.

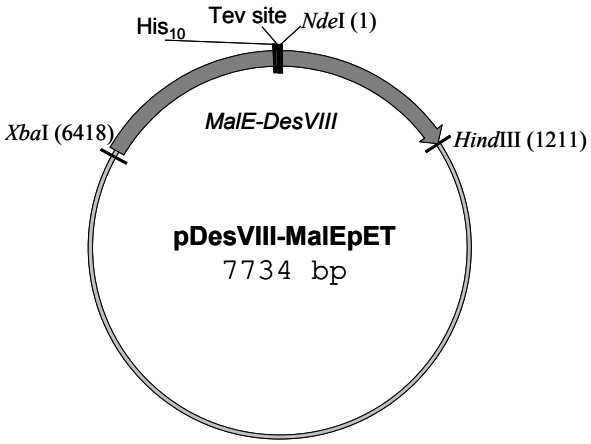

Figure 4-7: Expression plasmid pDesVIII-MalEpET used for the production of MalE-DesVIII fusion protein.

Purification of DesVIII-4 Protein. Cell pellet prepared in the previous step was thawed and resuspended in 150 mL of the lysis buffer (50 mM sodium phosphate pH 8, 0.3 M NaCl, 10% glycerol, 5 mM imidazole) containing 0.3 mM PMSF and 1 mg/mL lysozyme. After incubation on ice for 30 min, cells were disrupted by sonication (36 pulses of 10 sec sonication with 20 sec cooling intervals between pulse), and cell debris was removed by centrifugation.

Crude extract was incubated with 20 mL of Ni-NTA resin (bed volume) at 4 ^{o}C for 2 h, and the matrix was loaded into an empty column. After the removal of the flow-through, weakly bound proteins were eluted with 200 mL of the lysis buffer, followed by 150 mL of the lysis buffer containing 15 mM imidazole, and 100 mL of the lysis buffer containing 30 mM imidazole. Bound proteins were then eluted with the lysis buffer containing 250 mM imidazole, and the fractions containing the desired protein (88.9 kDa), as judged by SDS-PAGE, were combined. A recombinant His_6-tagged Tev protease (prepared by Peng Gao) was

added to the pooled fractions to a concentration of 15 μg/mL, and the solution was dialyzed against 1 L of 50 mM sodium phosphate, pH 8, 0.1 M NaCl, 10% glycerol with two changes of buffer overnight.

The complete digestion of MalE-DesVIII was run concurrently with dialysis overnight and confirmed by the presence of two major bands, 42.2 kDa for DesVIII-4 and 46.7 kDa for MBP (MalE), on SDS-PAGE. The dialyzed solution was incubated with Ni-NTA resin as in the previous step. Proteins were first eluted with 15 mM imidazole and followed by an elution with 250 mM imidazole. The fractions containing MBP (250 mM imidazole) were discarded, and the flow-through and 15 mM imidazole fractions containing DesVIII-4 were combined and dialyzed against the same dialysis buffer as above. While the major protein component is DesVIII-4, this sample still contains some MBP and the fusion protein as indicated by the Western blot analysis.

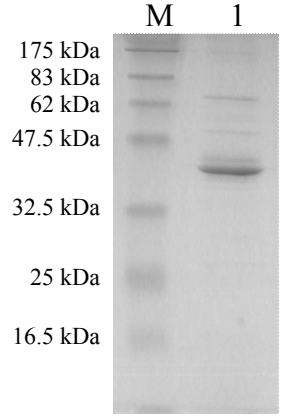

Figure 4-8: SDS-PAGE of purified DesVIII-4 (lane 1, major band of approximately 42 kDa). Lane M is prestained molecular weight marker and bands are as follows: *E. coli* MBP-β-galactosidase, 175 kDa; *E. coli* MBP-paramyosin, 83 kDa; bovine glutamic dehydrogenase, 62 kDa; rabbit aldolase, 47.5 kDa; rabbit triosephosphate isomerase, 32.5 kDa; bovine β-lactoglobulin A, 25 kDa; chicken lysozyme, 16.5 kDa.

After the dialysis, DesVIII-4 was concentrated and loaded onto an amylose column (15 mL bed volume). Unbound DesVIII-4 was eluted with 25 mM Tris-HCl, pH 8, 0.1 M NaCl, 10% glycerol, concentrated, and stored in the same buffer (Figure 4-8). The fractions eluted with 20 mM maltose contained MBP. The concentration of DesVIII-4 is 5.5 mg/mL as estimated by the Bradford assay and it is free of MBP and the fusion MalE-DesVIII according to the Western blot. A preliminary native molecular weight determination by size exclusion chromatography (as in Section 4.2.4) resulted in several peaks. The major one corresponds to 56.8 kDa, and the second major peak is the aggregated protein which was eluted with the void volume.

4.2.21 Large Scale DesVII/DesVIII Assay and Product Purification

To confirm the structure of DesVII product, a large scale reaction was ran as above (Section 4.2.17) with 50 μmol of **65**, 54 μmol of **22**, 0.36 μmol DesVII-C-His, and approximately 0.2–0.3 μmol DesVIII-4 in a total volume of 17 mL of 50 mM Tris-HCl buffer (pH 9) for 9 h. The products were extracted twice with an equal volume of chloroform; the combined organic layers were evaporated; and the residue was dried under vacuum. NMR spectra (^{1}H, ^{13}C and COSY) of the resulting product (25 mg, 55 μmol) were taken. They were in good agreement with the previously reported spectral data.[32, 42]

^{1}H NMR (500 MHz, $CDCl_3$) of YC-17 (**3**): δ 6.75 (1H, dd, J = 16.0, 5.5 Hz, 9-H), 6.43 (1H, dd, J = 15.5, 1.5 Hz, 8-H), 4.95 (1H, ddd, J = 8.5, 6.0, 2.5 Hz, 11-H), 4.25 (1H, d, J = 7.5 Hz, 1′-H), 3.59 (1H, d, J = 10.5 Hz, 3-H), 3.49 (1H, dqd, J = 12.0, 6.0, 2.0 Hz, 5′-H), 3.25 (1H, dd, J = 10.0, 7.0 Hz, 2′-H), 2.86

(1H, dq, J = 10.0, 6.5 Hz, 2-H), 2.62 (1H, dqq, J = 12.5, 7.0, 1.0 Hz, 10-H), 2.54 (2H, 2 sets of m, 3′-H and 6-H), 2.31 (6H, s, NMe_2), 1.71 (2H, m, 5a-H and 12a-H), 1.69 (1H, m, 4′ax-H), 1.56 (1H, m, 12b-H), 1.46 (1H, ddd, J = 14.0, 12.5, 4.0 Hz, 5b-H), 1.41 (3H, d, J = 7.0 Hz, 2-Me), 1.26 (1H, m, 4′eq-H), 1.23 (3H, d, J = 6.0 Hz, 5′-Me), 1.22 (1H, m, 4-H), 1.19 (3H, d, J = 7.0 Hz, 6-Me), 1.10 (3H, d, J = 6.5 Hz, 10-Me), 1.01 (3H, d, J = 6.5 Hz, 4-Me), 0.91 (3H, t, J = 7.5 Hz, 12-Me).

^{13}C NMR (125 MHz, $CDCl_3$): δ 205.29 (C-7), 175.20 (C-1), 146.83 (C-9), 126.00 (C-8), 105.00 (C-1′), 85.81 (C-3), 73.68 (C-11), 70.35 (C-2′), 69.42 (C-5′), 65.94 (C-3′), 45.11 (C-6), 44.10 (C-2), 40.27 (NMe_2), 37.85 (C-10), 34.12 (C-5), 33.51 (C-4), 29.71 (C-4′), 25.17 (C-12), 21.14 (5′-Me), 17.70 (6-Me), 17.40 (4-Me), 16.06 (2-Me), 10.30 (12-Me), 9.58 (10-Me).

The sample isolated from a large scale reaction contained a small amount of aliphatic impurity displaying signals as following ^{1}H NMR: δ 0.83 (m), 1.3 (t and m), 2.35 (broad d) and ^{13}C NMR: δ 28.57). Further purification of this compound using silica gel chromatography led to the recovery of only 3.5 mg of pure **3**.

4.2.22 HPLC Assay of DesVII/DesVIII Activity

Reactions in 50 or 100 μL scale were carried out to assay the DesVII/DesVIII activity as previously described for the TLC assays. The protein components were removed from the reaction mixture by filtering through a Microcon YM-10 centrifugal device. The filtrate was then chromatographed on an HPLC reverse phase analytical C_{18} column (4.5×250 mm, 5 μm). Either

Econosil C_{18} (Alltech, Deerfield, IL) or Microsorb-MV C_{18} (Varian, Palo Alto, CA) was used, both showing similar elution profiles. Data summarized in Table 4-2 was obtained using Microsorb-MV C_{18} column. Two alternative solvent systems were used for elution with flow rate of 1 mL/min. The detector was set at 235 nm.

1. Using Ammonium Acetate Buffer. Compounds were eluted isocratically with 50% acetonitrile in 0.057 M ammonium acetate. In this system, the retention time for **65** was 6.4 min and that for the product **3** (as a broad peak) fluctuated between 17.5 and 19.0 min, depending on its concentration when Econosil C_{18} column was used.

2. Using Triethylamine Buffer. The previously reported solvent composition was used.[43] Buffer A was 0.014 M triethylamine adjusted to pH 3 with trifluoroacetic acid (TFA), and buffer B was acetonitrile. Compounds were eluted with 26% B for 14 min, followed by 26–60% B gradient over 11 min, and then 60–100% B gradient over 10 min. This system gave better peak shape for the glycosylated compounds when Microsorb-MV C_{18} column was used. The retention times obtained under these conditions are shown in Table 4-2.

4.2.23 Far Western Blot Analysis of DesVII-DesVIII Interactions

The standard procedure was followed for Far Western blot analysis of protein interactions.[44] The sample containing the untagged DesVIII protein, DesVIII-1 (Section 4.2.16) was separated on SDS-PAGE, and proteins were transferred to Hybond-C Extra nitrocellulose membrane as described for Western blot (Section 4.2.1). After blocking and washing with appropriate buffers, the

membrane was exposed to 10 mL of a probe dilution buffer containing His_6-tagged DesVII (40 μg/mL) at 4 ^oC for approximately 16 h. The membrane was then washed in PBS buffer[44] and probed with an anti-histidine antibody using SuperSignal® West HisProbe™ kit from Pierce (Section 4.2.13). Chemiluminescence detection was used.

4.3 RESULTS AND DISCUSSION

4.3.1 Place of DesVII Among Other Glycosyltransferases

The *desVII* gene product was first assigned as the desosaminyltransferase in the methymycin/pikromycin pathway based on its sequence similarity to other known glycosyltransferases.[18] It shares high sequence similarity to a number of glycosyltransferases involved in the biosynthesis of macrolide antibiotics, such as OleG1 and OleG2 of the oleandomycin pathway in *S. antibioticus* (50.5% and 53.5% identity, respectively),[45] TylM2 of the tylosin pathway in *S. fradiae* (51.8% identity),[46] and EryCIII of the erythromycin pathway in *S. erythraea* (50.7% identity) (Figure 4-9).[47] DesVII also exhibits varied sequence similarity to GtfA, GtfB, and GtfC of the chloroeremomycin (**101**) pathway, GtfD and GtfE of the vancomycin (**100**) pathway in *Amycolatopsis orientalis* (18.8–20.9% identity),[8] and NovM of the novobiocin (**102**) pathway in *Streptomyces spheroides* (32.6% identity).[11]

It should be noted that during the course of the study we discovered that the nucleotide sequence originally deposited in the GenBank (accession number AF079762) is missing a fragment coding for the protein residues 136 through 149 (AGA VAA QVT GAA HA), while the protein sequence (accession number

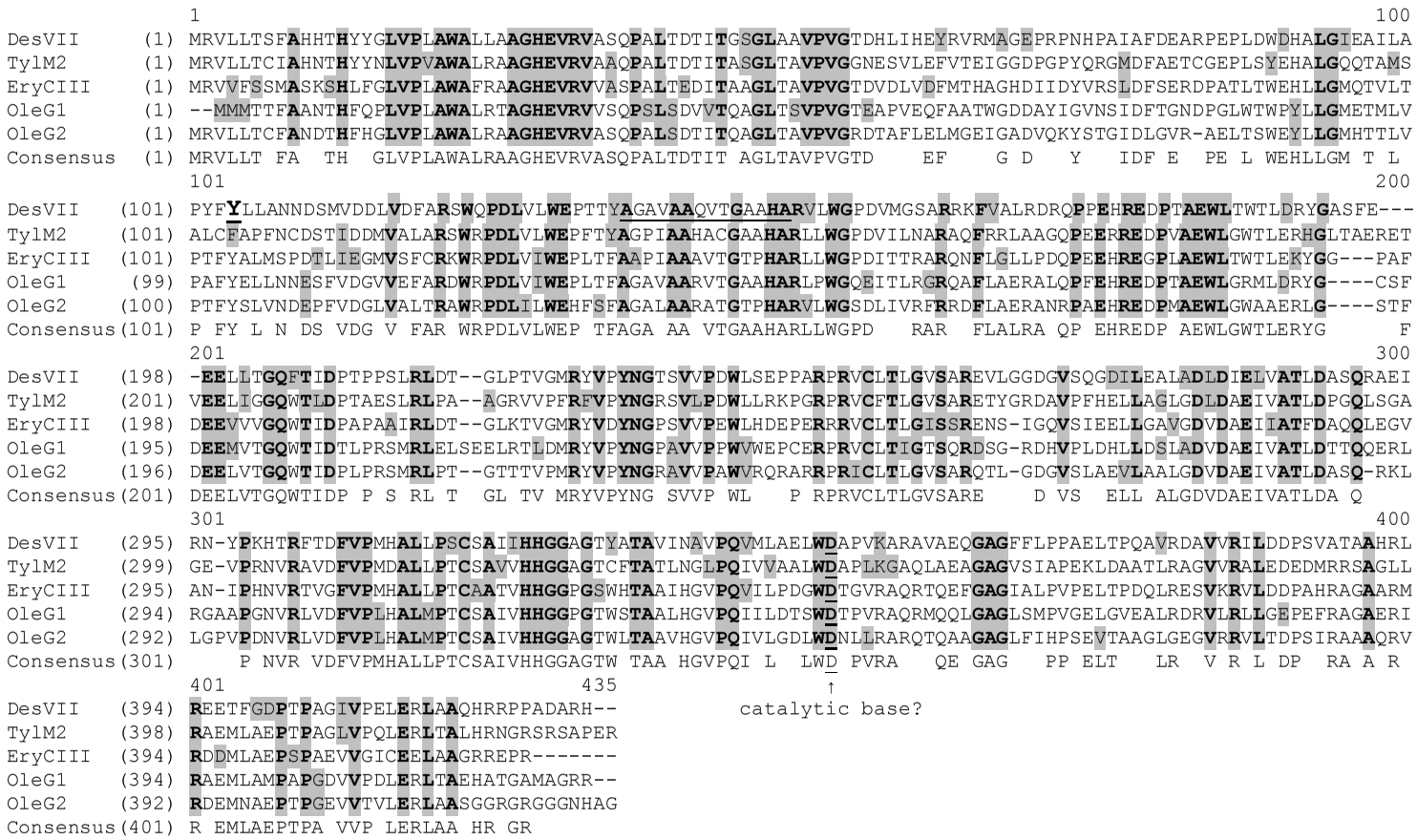

Figure 4-9: Sequence alignment of DesVII and its homologues involved in macrolide biosynthesis, TylM2 (accession number CAA57472), EryCIII (AAB84067), OleG1 (CAA05641), and OleG2 (CAB77098). Identical residues are shown in bold, and DesVII residues discussed in text are underlined.

AAC68677) does contain this fragment (Figure 4-9, underlined). Also, we discovered that the nucleotide sequence in the GenBank contains C (codon CAT) rather than T (codon TAT) at position 1784 (in the nucleotide sequence AF079762), which translates to a His rather than a Tyr at position 104 in the protein sequence (AAC68677) (Figure 4-9, underlined). This misassignment was confirmed by DNA sequencing. Thus, DesVII should be corrected to have Tyr-104 in its sequence, consistent with many other homologous glycosyltransferases containing an aromatic amino acid residue, usually tyrosine, at this position.

According to the CAZy database (Carbohydrate-Active enZYmes), DesVII has been placed in the glycosyltransferase family GT1. This family of proteins contains, among others, many inverting glycosyltransferases involved in the biosynthesis of secondary metabolites, including macrolides (*e.g.*, tylosin, erythromycin, oleandomycin, spinosin), aromatic polyketides (*e.g.*, daunorubicin, novobiocin, urdamycin, mithramycin), and nonribosomal peptides (*e.g.*, vancomycin, chloroeremomycin).[48-50] This classification is primarily based on the similarities in amino acid sequence, and it is useful for deducing structural and mechanistic insights, such as whether it is an inverting or a retaining glycosyl-transferase, how it evolutionarily related to other glycosyltransferases, and possible folds of its three-dimensional structure.

A complementary system for classification of glycosyltransferases has also been developed based on the known X-ray structures of several such proteins.[51] According to this system, all of the diverse glycosyltransferases can be assigned to one of the two superfamilies or protein folds, GT-A and GT-B.[52, 53]

Members of the GT-A superfamily include SpsA,[54] LgtC,[55] bovine α-1,3-galactosyltransferase,[56] bovine β4GalT1,[57] rabbit GnT1,[58] and human β-1,3-glucuronyltransferase,[59] while those belonging to superfamily GT-B include bacteriophage T4 β-glucosyltransferase BGT,[60] MurG,[61] and GtfB.[62] Based on the amino acid sequence alignment of DesVII with glycosyltransferases with known tertiary structures, DesVII should be a member of the superfamily GT-B. This superfamily is extremely diverse and consists of prokaryotic and eukaryotic enzymes with various donor and acceptor substrates. Most of the prokaryotic enzymes that glycosylate secondary metabolites belong to this superfamily. Although there are only four published crystal structures of the GT-B superfamily members to date, BGT, MurG, GtfB, and most recently GtfA,[63] all of them contain a two-domain structure with the active site located in the deep cleft between the two domains. The *N*-terminal domain binds the acceptor, and the *C*-terminal domain binds the sugar donor. Unlike the GT-A superfamily members, the GT-B superfamily enzymes lack a conserved DXD metal ion binding motif for binding the sugar-nucleotide, since the majority of the GT-B superfamily members are not metal-dependent. Instead, it is proposed that the Rossmann-like motif of the *C*-terminal domain is part of the binding site for the sugar donor substrate.[53] The two-domain structure of GT-B glycosyltransferases is believed to be the determinant controlling the structural diversity of the final products.

The closest homologue of DesVII with a known crystal structure is GtfB (19.8% identity), catalyzing the attachment of a glucose residue to the vancomycin aglycone (Figure 4-1). The analysis of GtfB crystal structure and

site-directed mutagenesis revealed that its residue Asp332 likely plays a role as the general base in the glycosyl transfer reaction. This residue is strictly conserved among the macrolide glycosyltransferases, including DesVII (Figure 4-9), and is expected to have the same function in their respective catalytic mechanisms.

4.3.2 Disruption of *desVII* in *S. venezuelae*

To verify the assigned function of *desVII* gene, a *S. venezuelae* KdesVII mutant, in which *desVII* gene is replaced by the thiostrepton resistance gene, was constructed as described in Chapter 2 following a literature procedure.[19] The disruption plasmid pDesVII-K2 (derived from the pKC1139 vector) was constructed, and its sequence was verified by another member of our group, Charles Melançon. It was used for a homologous recombination between the donor strain, *E. coli* S17-1 which is the host of this plasmid, and the wild type *S. venezuelae*. Double-crossover mutants were selected based on their phenotype as apramycin sensitive (Apr^S, indicates the departure of pKC1139 containing *desVII* gene) and thiostrepton resistant ($Thio^R$, confirms the incorporation of thiostrepton resistance gene in place of *desVII* in the chromosome). The resulting mutant, KdesVII-2, was fermented as described in Chapter 2 using the 12-membered ring fermentation conditions,[64] and its macrolide production was compared to that of the wild type *S. venezuelae* using TLC.

As expected, KdesVII-2 mutant did not produce any glycosylated macrolides. Only aglycone, 10-deoxymethynolide (**65**), and its hydroxylated derivatives, methynolide (**74**) and neomethynolide (**75**), were detected. This

result confirmed that DesVII is indeed the glycosyltransferase responsible for the desosaminylation of 10-deoxymethynolide (**65**).

4.3.3 Preparation of DesVII Substrates and Product

One of the obstacles to study the glycosyltransferases involved in the biosynthesis of secondary metabolites is the difficulty of obtaining their substrates. I am greatly indebted to our research group members, current and former, for the chemical and enzymatic syntheses of TDP-D-desosamine (**22**), TDP-D-mycaminose (**25**), TDP-β-L-rhamnose (**109**), and TDP-α-L-rhamnose (**108**), which are donor substrates or potential substrates of DesVII (see Section 4.2).

Acceptor substrate, 10-deoxymethynolide (**65**), was isolated from the fermentation broth of *S. venezuelae* KdesI-80 mutant, which lacks a key enzyme to complete the desosamine biosynthesis leading to the accumulation of aglycone (Chapter 2).[29] The medium optimized for a 12-membered ring macrolide production was used in this case.[64] An alternative substrate, 14-membered narbonolide (**64**), was provided by a group member Dr. Haruko Takahashi by fermenting the same mutant, KdesI-80, under the 14-membered macrolide production conditions.

To facilitate the analysis of the DesVII reaction, a sample of one of its products, YC-17 (**3**), was needed. YC-17 is a substrate of P450 hydroxylase, PikC, also known as PicK, that converts YC-17 into methymycin and neomethymycin *via* hydroxylation at C-10 or C-12 position, respectively.[31, 32, 65, 66] PikC also catalyzes the hydroxylation of narbomycin (**5**) at

C-12 position to give pikromycin (**4**). Inactivation of the *pikC* gene should result in the accumulation of YC-17 (**3**) (or narbomycin (**5**) under different conditions) in the *S. venezuelae* cultures.[31]

The isolation of YC-17 (**3**) from the fermentation broth of *S. venezuelae* mutant AX906 has been reported in an early collaborative effort with Professor David Sherman.[31] The strain *S. venezuelae* AX906 was supplied to us by Professor Sherman. Despite the fact that the strain has the correct phenotype (Kan^R, cell shape is also as expected for *S. venezuelae*), we failed to detect any methymycin- or pikromycin-related macrolide products in the fermentation broth of this mutant. Multiple attempts were made; sixteen individual colonies were isolated, but none of them produced the desired macrolide compound. Therefore, we decided to use a different approach.

It was reported that the addition of the monooxygenase inhibitor, xanthotoxin, to the cultures of *S. venezuelae* could force the accumulation of 10-deoxymethynolide (**65**).[40] We expected that some amount of YC-17 (**3**) may also be produced under this condition. Unfortunately, when xanthotoxin was added to *S. venezuelae* culture in a vegetative media, no YC-17 (**3**) was detected in the chloroform extracts by TLC analysis. But the amounts of methymycin (**1**) and neomethymycin (**2**) were significantly reduced as compared to culturing in the absence of the inhibitor (estimated to be about 20% of that produced in the control reaction). An additional component, identified to be 10-deoxymethynolide (**65**), was also detected. It is possible that the presence of an inhibitor has some effect

on the upstream genes or gene products involved in the sugar biosynthesis or attachment.

In order to overcome the problem of obtaining YC-17 (**3**), we opted to prepare a *pikC* mutant by ourselves. The *S. venezuelae* mutant in which the *pikC* gene was replaced with kanamycin resistance gene (*neo*) was generated using a homologous recombination between disruption plasmid pPikC-K and the genomic DNA of the wild type strain. Unlike AX906 mutant in which *pikC* was disrupted by insertional inactivation *via* double crossover, the KpikC-2 strain has *pikC* disrupted through a deletional inactivation, namely, 1,127 bp fragment of *pikC* gene (out of 1,251 bp total) is replaced with *neo*. KpikC-2 produced YC-17 (**3**) as the major product in a vegetative medium (approximately 300 mg from 4 L culture), with the second major component being narbomycin (**5**, 40 mg, not purified). A sufficient amount of YC-17 (**3**) (100 mg) was obtained from the separation of the crude extract by silica gel chromatography.

4.3.4 Purification of DesVII-C-His Protein

4.3.4A Purification of DesVII-C-His Protein

A former member of our group Dr. Lishan Zhao made the first attempt to detect DesVII activity as glycosyltransferase using the crude extracts of *E. coli* BL21 containing pHC29. The plasmid pHC29 was originally prepared by another former group member Dr. Huawei Chen by cloning the *desVII* gene into the *Nde*I-*Xho*I restriction sites of pET24b(+).

In this study, plasmid pHC29 was used to transform the expression host *E. coli* BL21. Upon the induction with IPTG, DesVII-C-His was produced by

E. coli BL21/pHC29. It was purified using affinity chromatography with Ni-NTA agarose to near homogeneity, and the resulting protein was stored in 50 mM Tris-HCl buffer (pH 8) supplemented with 10% glycerol as 25 or 100 mg/mL solution. Attempts to further purify DesVII-C-His using FPLC equipped with MonoQ or Superdex columns led to a significant loss of the protein due to precipitation and, therefore, were abandoned.

4.3.4B Native Molecular Weight Determination of DesVII-C-His

The composition of the native DesVII-C-His was determined using gel filtration chromatography on Superdex 200 HR 10/30 column. Fitting to the calibration curve constructed using protein molecular weight markers, a molecular weight of approximately 108 kDa for DesVII-C-His was deduced. Thus, the native DesVII-C-His is likely a homodimer under the experimental conditions (calculated molecular weight of the monomer is 47.5 kDa). It should be noted that a significant amount of protein (estimated 25%) eluted as an aggregated fraction with a molecular weight of approximately 1,379 kDa.

4.3.4C Attempts to Refold DesVII-C-His

One possible reason that the early experiments to detect DesVII activity were unsuccessful is due to the misfolding of DesVII during protein synthesis. To test this contention, we decided to try to refold DesVII-C-His by denaturing it first and then renaturing it while it is still bound to the resin.[67] However, this attempt failed due to the protein precipitation upon the addition of the renaturing buffer and was later abandoned. Therefore, a protein sample of DesVII-C-His obtained as described above was used for further studies.

4.3.5 Possible Problems and Solutions to Detect DesVII Activity

Dr. Lishan Zhao made the first attempt to detect DesVII activity as a glycosyltransferase, however, he was unsuccessful to show the activity of the enzyme. Several reasons are conceivable for the absence of DesVII activity reported by Dr. Lishan Zhao.

1. Inappropriate assay conditions were used. Dr. Zhao had tried many modifications, such as using different buffers (potassium phosphate or Tris-HCl), different pH (6.2, 7.0, 8.1, or 9.0), different divalent metal cations ($MgCl_2$ or $MnCl_2$), and different organic solvent for the preparation of aglycone stock solution (ethanol or DMSO). The introduction of additional components, such as BSA at 1 mg/mL or Triton X-100 at 0.2% v/v, was also attempted. However, none of these changes seemed to improve the assay outcome. Thus, a more systematic check of assay conditions may be necessary.
2. DesVII is in an inactive form. It may be misfolded during expression or denatured during purification. Since the denaturing/renaturing attempt described above was unsuccessful, other procedure to refold the purified protein will be tested. Using an *in vitro E. coli* transcription/translation system to produce DesVII, or to express *desVII* in the *S. lividans* host may also be worth a try.
3. The postulated acceptor substrate of DesVII may be incorrect. The fact that purified DesVII failed to couple TDP-D-desosamine to the putative macrolactone substrate, 10-deoxymethynolide (**65**), has prompted us to

consider a new hypothesis in which a linear polyketide attached to the acyl carrier protein (ACP) domain of the methymycin polyketide synthase, instead of 10-deoxymethynolide (**65**) itself, may be the true substrate of DesVII. This hypothesis is clearly provocative and needs to be tested.

4. An additional protein component may be required for a successful glycosyl transfer. As mentioned in Chapter 1, the originally proposed function of *desVIII* is to code for a tautomerase in the TDP-D-desosamine biosynthesis. It later became apparent that the tautomerization step is not necessary for the formation of TDP-D-desosamine (Chapter 2).[68] However, *desVIII* disruption experiments demonstrated that DesVIII is indeed essential for the biosynthesis of methymycin/neomethymycin and most likely participates in the desosaminylation of aglycone step(s). The initial efforts to examine whether it has any effect on DesVII activity will be described in this section. The in-depth study of DesVIII involvement in glycosylation will be presented later in this chapter.

4.3.5A Co-expression of desVII and desVIII in E. coli

In order to test the hypothesis of whether DesVIII is required for DesVII activity, attempts were made to clone and express *desVIII* and *desVII* genes together in *E. coli*. These genes are located next to each other on the chromosome of *S. venezuelae*, *desVIII* upstream of *desVII*. They are in frame and are separated by a 147-bp stretch of DNA. The amplification of a 2,562-bp fragment of pLZ4 cosmid containing both genes proved to be an obstacle. No desired DNA product was obtained in PCR reaction when different annealing temperatures and DNA

polymerases (*Pfu* or Advantage cDNA polymerases) were used. After several attempts this strategy was abandoned.

4.3.5B Glycosyltransferase Assay with DesVII-C-His

The recipe of this assay was developed by Dr. Lishan Zhao as described in Section 4.2.5. The protein used was DesVII-C-His (pure or crude) with varied concentration. The negative controls contained the same volume of the protein dialysis buffer in place of DesVII-C-His. No product was detected by TLC after two hours of incubation at 29 ^{o}C under the assay conditions.

4.3.5C Assays Using Crude Protein Extracts of Wild Type S. venezuelae and Its KdesVIII-92 Mutant

Since no activity was detected with DesVII-C-His alone, crude protein extracts of the wild type *S. venezuelae* and its KdesVIII-92 mutant were also added to the assay mixture to determine whether additional components are required for activity. Crude extracts of each strain were partially purified by ammonium sulfate fractionation. Two fractions precipitated with 0–60% and 60–90% ammonium sulfate were collected. These fractions were added to the assay mixture either as the protein component alone (10–20% v/v) or together with DesVII-C-His. The chloroform extracts of the incubated assay mixtures (2 h, 29 ^{o}C) were examined by TLC analysis.

In addition to the unreacted substrate, 10-deoxymethynolide (**65**), the production of a new compound was observed when the 60–90% ammonium sulfate precipitate fraction from either wild type or mutant *S. venezuelae* was used, with or without the addition of DesVII-C-His. This new compound is more

polar than **65** with an R_f value expected for YC-17 (**3**). However, the same compound was also detected when just the disruption buffer was added to the assay mixture. This compound must therefore be a decomposition product of 10-deoxymethynolide (**65**) and is likely resulted from a Michael addition of DTT at the C_8-C_9 double bond conjugated to the ketone moiety in **65**. The structure of this Michael adduct was confirmed by ^{1}H NMR. No other products were detected.

4.3.5D Expression of desVII in S. lividans: 1st Attempt

Expression of *Streptomyces* genes in *S. lividans* proved to be useful in many cases when proteins could not be produced or properly folded in *E. coli*. Therefore, we attempted to express *desVII* in *S. lividans* 1326.

The gene *desVII* was cloned in the plasmid vector pIJ702 routinely used for the expression of genes in *Streptomyces lividans*.[22, 36] Vector pIJ702 contains thiostrepton resistance (Thio^R) marker and melanin operon (*melC*) of *S. antibioticus*, but the latter is disrupted when *desVII* is inserted. The construct based on the pIJ702 vector was designed to have DesVII fused with a *C*-terminal histidine tag (desVII-Sl-C-His) when expressed. The transformation of *S. lividans* 1326 protoplasts with this construct resulted in the formation of thiostrepton-resistant colonies phenotypically similar to *S. lividans* 1326, *i.e.* covered in white spores and producing dark purple pigment. However, the *S. lividans* control strain without the plasmid also produced a lower, but still significant, number of colonies. The isolation of the plasmid DNA from the selected clones for agarose gel analysis was not successful. The analysis of crude proteins isolated from

these clones using anti-histidine detection system did not result in any visible bands on the Western blot. Thus, no DesVII-C-His fusion protein was produced.

The difficulties with this approach result from the use of pIJ702 vector. Since it cannot be used to transform *E. coli*, all the steps after ligation have to be performed in *S. lividans*. Therefore, isolation of the constructed plasmid was more problematic. Since the negative control strain also produced a few background colonies, it was difficult to tell whether the selected clones contained the plasmid (pIJ702 with or without the insert), and if they did, whether these plasmids were the correct ones. Thus, we decided to take a different approach to express *desVII* in *S. lividans*, where we could verify the constructs before introducing them into the host.

4.3.5E Expression of desVII in S. lividans: 2nd Attempt

A new strategy was developed for *desVII* expression in *S. lividans*. First, pUC119 vector will be incorporated into the pIJ702 vector to simplify cloning procedures and verification of the resulting constructs. This pUC119 portion in this hybrid vector can be later excised, and the resulting plasmid self-ligated prior to the transformation of *S. lividans*. Second, in this construct, *desVII* will be cloned downstream of the *melC1* gene in the pIJ702 vector, instead of replacing it as in the previous experiment. Gene *melC1,* in addition to *melC2*, in the pIJ702 plasmid is derived from the *melC* operon of *S. antibioticus*. While *melC2* translates into tyrosinase MelC2, the protein encoded by *melC1* is involved in the activation and secretion of MelC2.[69] Together, MelC1 and MelC2 are necessary for the production of melanin pigment. Thus, incorporation of *desVII*

downstream of *melC1* to replace *melC2* should result in the secretion of DesVII by *S. lividans*. This would significantly simplify the analysis of proteins produced by *S. lividans*.

The plasmid pSB01, derived from the pIJ702 vector harboring pUC119 fragment, *melC1*, *desVII*, and the kanamycin resistance marker *neo*, was constructed. It was used to transform the protoplasts of *S. lividans* TK64, and the transformants were selected based on their resistance to kanamycin. The success of transformation was confirmed by PCR analysis of the DNA preparations from selected clones. The extracellular (supernatant from the centrifuged culture) and cell proteins (pellet) were analyzed by SDS-PAGE. A band of approximately 47.2 kDa was observed in the supernatant fractions, implicating the presence of DesVII-C-His protein in these fractions.

The glycosyltransferase assay was performed as described above, with the addition of the concentrated extracellular fractions. TLC analysis of the chloroform extract from the reaction mixture revealed the presence of unreacted substrate 10-deoxymethynolide (**65**) in the mixture but no YC-17 (**3**) product. Evidently, protein expression in *S. lividans* did not provide active DesVII, at least when tested under the above assay conditions.

4.3.5F Production of DesVII Using in vitro Transcription/Translation System

In search for active form of the glycosyltransferase DesVII, the production of DesVII by Rapid Translation System (RTS) which uses *E. coli* lysate for gene overexpression *in vitro* was also attempted. We used plasmid pHC29 as the template for transcription in this experiment. In fact a gene cloned into any of the

pET vectors can be used, since the pET vector satisfies all of the requirements for DNA templates of this system.

Template for the synthesis of green fluorescent protein (GFP) was used as the positive control. After proper incubation, GFP was produced in the positive control (by Western blot analysis and fluorescence at UV_{365}); however, no product was detected by SDS-PAGE or the Western blot analysis when pHC29 was used as the template. Attempts to purify (or concentrate) DesVII sample using Ni-NTA agarose failed to yield the expected protein. Therefore, RTS did not solve the problem to obtain DesVII. Consequently, this strategy was abandoned.

4.3.5G Summary

This section summarized our initial attempts to detect glycosyltransferase activity of DesVII. Significant efforts were directed to the production of active DesVII by various systems or methods. These include using *E. coli* and *S. lividans* as host cells, using *E. coli* extracts as protein source in assays, and refolding the purified DesVII to renature the protein. We have also tested crude protein extracts of *S. venezuelae* and its KdesVIII mutant as possible sources of glycosyltransferase activity. This test was also carried out in the presence of purified DesVII. However, none of these attempts were successful. Reflecting upon the setbacks of these experiments, we have come up with a fundamentally different hypothesis regarding the chemical nature of the acceptor substrate of DesVII. Our efforts to test this hypothesis are the subject of the following section.

4.3.6 New Hypothesis: Glycosylation Catalyzed by DesVII Occurs Prior to the Detachment of Polyketide Chain from PKS

4.3.6A Background and Strategy

It has been widely accepted that the substrates for a macrolide glycosyltransferase include an NDP-sugar donor and a macrolactone acceptor. In the case of DesVII, they are TDP-D-desosamine (**22**) and 10-deoxymethynolide (**65**). Enough evidence has been accumulated supporting sugar in its NDP- form as a substrate for all glycosyltransferases. In fact, the amino acid sequence of known macrolide glycosyltransferases all contain an NDP-binding domain. The identity of the aglycone substrate, however, has never been established by any *in vitro* study.

We eliminated the methynolide (**74**) and neomethynolide (**75**) as possible substrates since it has been shown that hydroxylation of macrolide happens after the glycosyl transfer step (Section 1.5). While 10-deoxymethynolide (**65**) would be the most reasonable acceptor in DesVII-catalyzed reaction, the failure of DesVII to achieve the coupling of TDP-D-desosamine to **65** under our assay conditions imposed serious challenge to the above assumption. We then considered the linearized form of **65** with the carboxyl group exposed or substituted in coenzyme A (CoA) thioester or other ester forms as the aglycone substrate. This hypothesis, however, would involve a considerable modification of the well-studied polyketide biosynthesis, particularly, the cyclization step by the thioesterase (TE) domain of polyketide synthase. It is thus more probable that the aglycone substrate is the linear form of 10-deoxymethynolide (**65**) which is

still attached to the acyl carrier protein (ACP) domain of PKS prior to being cleaved by the TE domain through a cyclization reaction.

This postulation is not without a precedent, since it is somewhat analogous to the *N*-acylation of one of the N_ε amino group of asparagine (Asn_1) of the growing nonribosomal peptide chain tethered to the peptidyl carrier protein (PCP) domain of the mycosubtilin nonribosomal peptide synthetase (NRPS) cluster. It is thought that the attached acylating β-amino-acyl-C_{18} chain is generated from a fatty acid myristate *via* the catalyses of several other domains of NRPS and is transferred onto Asn by the action of the condensation (C) domain.[70] In contrast to the mycosubtilin case, in the NRPS clusters of other *N*-acylated nonribosomal peptides the corresponding acyltransferase is not a part of the cluster.[71](p.214)

Accordingly, our hypothesis is that DesVII catalyzes the glycosylation at C-3 hydroxyl group of the growing polyketide chain attached to ACP_5 domain of PikAIII PKS (**23**, Figure 4-10), followed by TE catalyzed cyclization, resulting in the production of 12-membered YC-17 (**3**). Chain extension catalyzed by another condensation unit prior to cyclization would lead to the formation of the 14-membered narbomycin (**5**). It is also possible that both hexaketide and heptaketide, attached to ACP_5 and ACP_6, respectively, can be glycosylated by DesVII. The glycosylated polyketide chain is then transferred to the serine residue of TE domain, cyclized by macrolactonization, and subsequently released.

To test this hypothesis we set out to (i) generate the linear form of 10-deoxymethynolide (**65**) in its CoA thioester form (**104**); (ii) attach it to the recombinant apo-ACP_5 domain *via* the action of phosphopantetheinyl transferase

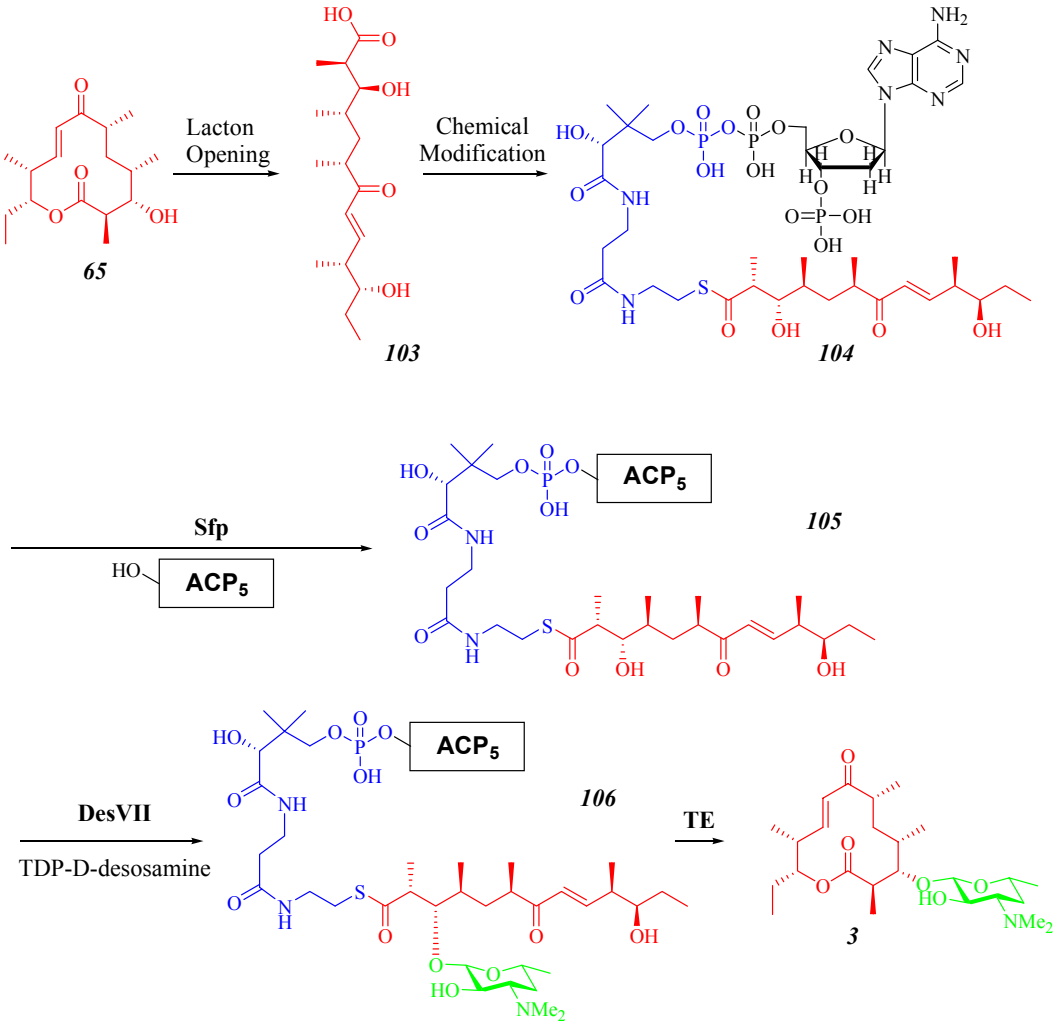

Figure 4-10: Experimental strategy to test the new hypothesis about DesVII acceptor substrate.

Sfp; (iii) incubate the resulting holo-ACP_5 (**105**) with DesVII under glycosylation conditions; (iv) treat the glycosylated product **106** with recombinant TE or an entire polyketide synthase PikAIV to cyclize the macrolactone. If our hypothesis is correct, this scheme, summarized in Figure 4-10, would result in the production and release of glycosylated macrolide YC-17 (**3**).

4.3.6B Preparation of Sfp

The *Bacillus subtilis* Sfp protein is part of the biosynthetic machinery responsible for the formation of lipopeptide antibiotic surfactin.[38] It activates the apo-forms of peptidyl carrier protein (PCP) domains of surfactin synthetase by transferring the 4′-phosphopantetheinyl (Ppant) moiety of CoA to the serine residue conserved in all PCPs, resulting in their respective holo-forms.[72] The thiol moiety of the Ppant linker is then loaded with a specific amino acid *via* acylation to form a thioester bond. The loaded amino acid is then used in subsequent nonribosomal peptide synthesis. Unlike other Ppant transferases (PPTases), Sfp can modify a wide variety of proteins including not only PCP substrates but also aryl carrier protein (ArCP) domain and ACP domain. It is also very flexible in regards to its CoA substrates. Sfp was shown to recognize not only CoA but also its acyl thioesters, such as acetyl, propionyl, butyryl, hexanoyl, isobutyryl, isovaleryl, acetoacetyl, benzoyl, and phenylacetyl thioesters,[73] as well as CoA aminoacylated by various amino acids.[74]

The crystal structure of the Sfp-CoA complex provides an explanation of Sfp's ability to use diverse CoA substrates.[75] It clearly shows that the pantetheinyl residue of CoA is flexible and is exposed to the solvent, and it makes no interactions with Sfp. The 3′-phospho-5′-ADP moiety of CoA on the other hand is buried in the binding pocket and makes contacts with Sfp necessary for binding of the substrate. Therefore, it is reasonable to expect that Sfp is able to convert apo-ACP_5 into holo-ACP_5 acylated with hexaketide (**105**).

The gene *sfp* from *B. subtilis* was expressed in *E. coli* BL21 as a *C*-terminal His_6 fusion protein using expression plasmid pET29-Sfp kindly provided by Dr. Christopher Walsh. Sfp (27.1 kDa) was purified to near-homogeneity using Ni-NTA affinity chromatography. It was later used in the radioassays with ACP_5 and labeled acetyl-CoA.

4.3.6C Preparation of apo-ACP_5

Acyl carrier protein domain ACP_5 is a part of module 5 of methymycin/ pikromycin PKS PikAIII (Figure 1-16). Its role in the biosynthesis of pikromycin is to serve as the reaction site for the 5^{th} chain extension reaction. After condensation, the hexaketide chain attached to its serine residue through Ppant linker is transferred to the keto synthase (KS) domain KS_6 and then condensed with ACP_6-linked methylmalonyl unit in the 6^{th} chain extension reaction. The resulting heptaketide is transferred to the TE domain of module 6, were cyclization takes place and subsequently the 14-membered macrolideproduct is released. When a truncated version of module 6 lacking the *N*-terminal section of KS_6 is produced by *S. venezuelae*, the hexaketide is transferred to TE directly from the ACP_5 domain.[76] Alternatively, the hexaketide may also be first transferred to ACP_6 without chain extension and then to TE.[77] Either way, the final cyclization product is a 12-membered macrolide. In order to test the new glycosylation hypothesis, we decided to produce a histidine-tagged ACP_5 in *E. coli* and to phosphopantetheinylate it with acyl-CoA using Sfp enzyme. Its competence as a substrate for DesVII will then be tested in reaction with TDP-D-

desosamine (**22**). The resulting adduct, if there are any, will be treated with TE (as a part of PikAIV) to release the final product.

Since PKS domains are components of a large protein molecule encoded by a single ORF rather than by separate ORFs, care must be taken when deciding on the boundaries of the separate domains for heterologous expression. The study done by Sherman and co-workers where ACP_5 domain was used as a part of hybrid PKS and retained its activity[78] helped us to design an expression plasmid ACP5-2/pET24b for the production of individual ACP_5.

ACP5-2/pET24b containing the fragment of *pikAIII* coding for the last 228 amino acids of PikAIII PKS was used for the production of ACP_5 (23.6 kDa) in *E. coli* BL21. Although, it was reported that the *E. coli* PPTases are capable of priming foreign ACP and PCP domains, the catalytic efficiency is low.[73] Hence, short induction times should result in the production of the majority of ACP in its apo form. Since our goal was to obtain apo-ACP_5, we harvested ACP_5-producing cultures 3 to 4 h after the induction with IPTG at 37 ^{o}C. The protein was purified to near-homogeneity using affinity (Ni-NTA agarose) and anion exchange (MonoQ) chromatography and used in the model radioactive assays with Sfp and labeled acetyl-CoA.

4.3.6D Model Reaction: Synthesis of Acetyl-holo-ACP_5

To verify that Sfp is capable of priming apo-ACP_5, we first tested whether radioactively labeled acetyl CoA [acetyl-^{3}H] can be recognized as the second substrate by Sfp. A radioassay developed by Walsh *et al.*[39] with minor modifications was used. Figure 4-11 shows a representative example of the Sfp

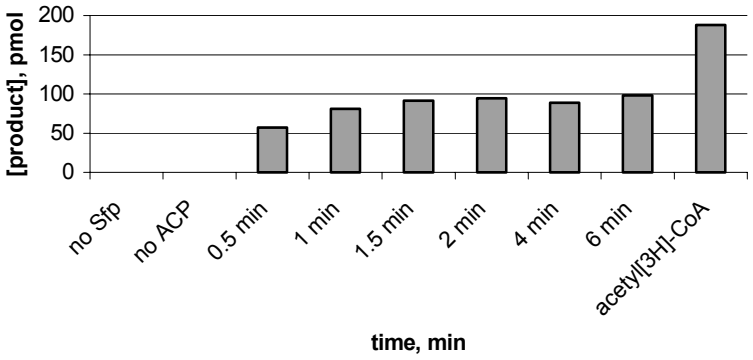

Figure 4-11: Radioassay: representative Sfp reaction progress data. First two bars are for negative control reactions without Sfp and without ACP_5-C-His, respectively, incubated for 2 min. The last bar is the labeled substrate (same amount as in the assay mixtures) in the scintillation cocktail.

radioassay data. We found that the reaction is complete within 2 min after the addition of Sfp, despite the fact that only 50% of the radioactivity added is present in the protein pellet. It appeared that only 50% of the acetyl CoA was used. This may be an indication that a significant amount of ACP_5 used in the assay was in the holo form. When ACP_5-C-His obtained from the culture induced by IPTG for 16 h at 24 ^{o}C was used, the conversion rates were even lower (5–10%), indicating that the majority of ACP_5 produced by cells which were harvested late was in its holo form. Another reason for the low yield of the modified protein might be a slow decomposition of acetyl CoA in the stock solution over the time, resulting in the lower amounts of substrate than assumed in the assay mixture.

Nevertheless, this model assay demonstrated that Sfp is capable of adding an acetyl-phosphopantetheinyl moiety to ACP_5-C-His. Based on the relaxed acyl-

CoA substrate specificity of Sfp reported by other researchers,[73, 74] we expect that **104** (Figure 4-10) may also be used as a substrate by Sfp.

4.3.6E Preparation of Acyl-CoA Substrate 22 for Sfp Reaction

The chemical synthesis of the linear polyketide **103** is a challenging task due to its complex structure containing six stereogenic centers. Thus, we planned an alternative approach to generate **104** from the readily available 10-deoxymethynolide (**65**). This approach involves opening the macrolactone by chemical hydrolysis of the ester linkage, selectively activating carboxylic acid by the condensation with isobutyl chloroformate, and coupling it with CoA. However, the hydrolysis of the ester linkage was unsuccessful despite multiple attempts. In fact, the double bond conjugated to the ketone which constitutes a Michael acceptor is more reactive toward the base under some of the conditions tried. Group member Dr. Chai-Lin Kao has nearly completed an alternative multistep chemical pathway to make compound **103** from **65**. The results of his work will be reported elsewhere.

4.3.6F Purification of TE Domain

To probe the "Linear substrate hypothesis" a heterologously expressed TE domain was produced. Early experiments have shown that the TE domain is capable of catalyzing the *in vitro* hydrolysis of the thioester linkage in *N*-acetylcysteamine derivatives of polyketides, and it can also catalyze *in vitro* lactonization of unnatural polyketides attached to the ACP domain when fused to an appropriate PKS module.[79] It has also been demonstrated that the excised epothilone TE domain catalyzes an efficient cyclization of the *N*-acetylcysteamine

thioester of the linear epothilone.[80] Therefore, it is reasonable to expect that the purified TE domain can promote the release of polyketide, glycosylated or not, from DesVII-treated **105** (**106**). It is our hope that a cyclization catalyzed by TE will also take place resulting in the formation of YC-17 (**3**) or 10-deoxymethynolide (**65**). However, it is possible that, in our system, hydrolysis may prevail over lactonization and a linear polyketide will be released. In any case, the release of polyketide from ACP will facilitate its characterization. It should be mentioned that TE of epothilone PKS can catalyze hydrolysis of cyclic epothilone C to its linear form, seco-epothilone C.[80] If the same is true for the methymycin case, the purified TE can potentially be used as a more convenient means to prepare the linear 10-deoxymethynolide substrate **103**.

Purification of TE Domain of Methymycin PKS. The fragment of *S. venezuelae* DNA containing the TE domain was amplified by PCR and cloned into the expression vector pET24b(+). Since TE is a component of the PikAIV and its boundary is not well defined, two plasmids were constructed, pTE-C-His and pTE(2)-C-His, for the production of two versions of TE of different length with *C*-terminal His_6 tag. The insert of pTE-C-His encodes TE-1 (contains 288 *C*-terminal amino acids of PikAIV plus 13 amino acids to introduce His_6 tag), and that of pTE(2)-C-His encodes TE-2 (299 amino acids of PikAIV). Both proteins were overproduced in *E. coli* BL21 and purified using nickel affinity chromatography. While the purified TE-1 (32.2 kDa) was a nearly homogenous protein as shown by SDS-PAGE, the purification of TE-2 (33.1 kDa) was not

very successful, and TE-2 was isolated as a major component contaminated with other proteins.

Both TE-1 and TE-2 were assayed towards hydrolysis of 10-deoxymethynolide (**65**). No visible depletion of **65** under the assay conditions was detected by TLC analysis. Therefore, it is apparent that TE does not catalyze hydrolysis of macrolide under the conditions tested. Nevertheless, it may still be useful in the hydrolysis of polyketide thioesters.

4.3.6G Generation and Characterization of Mutants Lacking TE Domain

Since obtaining **103** proved to be difficult, we opted for generating ACP-linked polyketide using an alternative *in vivo* approach. Specifically, our plan involves the replacement of the fragment of DNA encoding the TE domain of PikAIV, TEII (PikAV), and DesVIII in the genome of *S. venezuelae* with a fragment containing the kanamycin resistance gene, preceded by a DNA portion encoding for His_6 tag. Such a mutant, KTE, would lack the ability to synthesize and/or attach TDP-D-desosamine (the effect of *desVIII* disruption) and would also be unable to cyclize and release the hexapolyketide moiety attached to ACP_5 or ACP_6 domains (TE function). PikAIV PKS of such a mutant will be truncated at the *C*-terminus and fused with a polyhistidine tag to facilitate the detection and purification of this protein. The attachment of a heptaketide chain to its ACP_6 domain is also possible when the strain is grown under the 14-membered ring production conditions.

The detection of PikAIV mutant in the crude extracts of *S. venezuelae* KTE will be performed using Western blot analysis with an anti-polyhistidine

antibody, following a similar procedure reported by Xue *et al.*[76] However, the anticipated low yield of PikAIV may impose a problem for its purification. In addition, precautions must be taken to avoid accidentally removing the polyketide chain during the purification. It was our hope that a sufficient amount of the heptaketide-PikAIV adduct could be purified using Ni-NTA resin. It could then be used as a substrate in the glycosylation assays.

Despite the fact that we could not obtain the expected heptaketide-PikAIV adduct, *S. venezuelae* mutant KTE generated here, as well as its derivatives complemented with other genes, were important to our understanding of the glycosylation biosynthetic step as shown later in this section.

Preparation of KTE Mutant of S. venezuelae. The functional TE domain of PikAIV, defined by Tsai and coworkers, is shown in Figure 4-12 (underlined).[81] The *C*-terminal portion of *pikAIV* deleted in this study contains three catalytic residues of TE. The site of deletion begins at Gln1142 as indicated by an arrow. The ORF *pikAV* encodes thioesterase type II, TEII, whose function remains elusive, but unlikely plays a direct role in the polyketide biosynthesis. It

PikAIV: MTS……LTGLQLPPTVVFQHPTPVALAERISDELAERNWAVAEP
SDHEQAEEEKAAAPAGARSGADTGAGAGMFRALFRQAVEDDRYGEFLDVLAEASAFRP
QFASPEACSERLDPVLLAGGPTDRAEGRAVLVGCTGTAANGGPHEFLRLSTSF↓QEERD
FLAVPLPGYGTGTGTGTALLPADLDTALDAQARAILRAAGDAPVVLLGH**S**GGALLAHE
LAFRLERAHGAPPAGIVLV**D**PYPPGHQEPIEVWSRQLGEGLFAGELEPMSDARLLAMG
RYARFLAGPRPGRSSAPVLLVRASEPLGDWQEERGDWRAHWDLPHTVADVPGD**H**FTMM
RDHAPAVAEAVLSWLDAIEGIEGAGK

Figure 4-12: Underlined *C*-terminal portion of PikAIV is TE according to Ref. 81 with a catalytic triad (Ser1196, Asp1224, His1316) shown in bold. Symbol ↓ indicates the beginning of the chromosomal region removed in the KTE mutant of *S. venezuelae*.

was deleted in our experiment mainly to simplify the cloning procedures. Removal of *desVIII* would disrupt the glycosylation step as discussed in Chapter 2.

The mutant was generated through a homologous recombination between the disruption plasmid pTE-K and chromosomal DNA of the wild type *S. venezuelae* as previously described. The presence of the desired mutation in two strains with the expected phenotype (Kan^R, Apr^S), KTE-1 and KTE-2, was confirmed by a Southern blot hybridization analysis using DIG-labeled **KTE**, **"desVIII"**, and **neo** fragments. A hybridization of mutant DNA with **KTE** and **"desVIII"** probes resulted in the bands of approximately 6.9 and 4.7 kb, respectively, while a hybridization of wild type DNA resulted in 12.8 kb band with both probes. Only the mutant DNA contains a fragment hybridizing with the

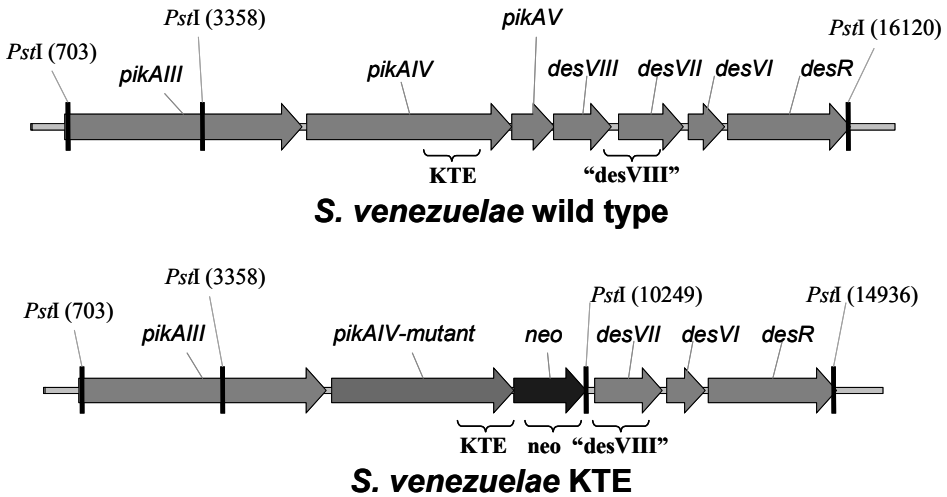

Figure 4-13: Chromosomal regions of interest in *S. venezuelae* wild type and KTE mutant. Restriction endonuclease *Pst*I was used for digestion of the genomic DNA, and the corresponding restriction sites are shown. The annotated fragments complementary to the DIG-labeled probes used for Southern hybridization are shown in bold.

neo probe (6.9 kb), and no such fragment is present in the chromosome of wild type *S. venezuelae*. These observations are consistent with the replacement of the *C*-terminal portion of *pikAIV* and the entire *pikAV* and *desVIII* in the chromosome of *S. venezuelae* as depicted in Figure 4-13.

As expected, no macrolide compounds were produced by KTE-1 and KTE-2 under either the 12- or 14-membered ring production conditions.

Analysis of Proteins in KTE-2 Mutant. Crude proteins were obtained from the KTE-2 mutant grown in a supplemented minimal medium and compared to the crude protein fraction of wild type *S. venezuelae* using SDS-PAGE and Western blot analysis. No apparent difference between the crude proteins isolated from these strains was detected. This result is not too surprising since PikAIV is likely a minor component of the proteome. In contrast, Western blot analysis with anti-polyhistidine detection is expected to highlight at least one, and possibly two, bands among KTE-2 proteins. One band should be approximately 121.0 kDa, corresponding to the mutated PikAIV-C-His. The other should be 99.5 kDa, representing the mutated PikAIV-C-His truncated at the *N*-terminal KS_6 domain. Having a phosphopantetheinyl linker would add 340 Da to this number, and the attachment of a hexaketide tail would add an additional 297 Da. To our disappointment, no bands of the indicated sizes were detected. In fact, the Western blot looked identical for both wild type and the KTE-2 proteomes.

Further efforts were made to facilitate the detection of PikAIV-C-His. For example, different conditions were used for culturing the strains. Growth in the SCM medium, used for the 12-membered ring production, is expected to give a

major band of 99.5 kDa, while in the 14-membered ring production medium, PGM, a major band of 121 kDa is expected. TSB medium, generally not used for secondary biosynthesis stimulation, was also tried. Cultures were grown for a long period of time, up to four days, and the crude protein fractions were partially purified using Ni-NTA agarose to simplify protein detection. A partial bacterial proteome extraction kit ProteoExtract^{TM} was also used to separate crude proteins based on their solubility properties. To increase the sensitivity of the Western blot analysis, chemiluminescence detection was used in place of the routine colorimetric detection. However, despite numerous attempts, no PikAIV-C-His was detected in the KTE-2 mutant strain.

Complementation of KTE-2 with desVIII. *S. venezuelae* KTE-2 was used as a host for the expression of *desVIII* using the *Streptomyces-E. coli* shuttle plasmid DesVIII/pAX617, which contains *desVIII* gene as an insert (see Chapter 2). The reintroduction of *desVIII* is expected to restore the desosamine biosynthesis and attachment in the host cells, while the macrolactone release would still be precluded due to the truncation of the *C*-terminal region of PikAIV and the deletion of PikAV. This strain was constructed to check whether a glycosylated polyketide can be detected to be attached to the ACP domain as predicted by the "Linear substrate hypothesis". While the incubation failed to produce positive results, the generated strain, desVIII/KTE, was found, as expected, to be deficient in macrolide production.

Expression of te in KTE-2 and in desVIII/KTE Strains. Gene *te*, which encodes TE-1, was expressed in the *S. venezuelae* KTE-2 and desVIII/KTE

Table 4-1: Analysis of Macrolide Production by *S. venezuelae* Mutants

S. venezuelae Strain	Natural Macrolide Production	Macrolide Production in Feeding Experiment
Wild type	methymycin, neomethymycin	10-deoxymethynolide, methymycin, neomethymycin
KTE-2	No macrolides produced	10-deoxymethynolide, methynolide, neomethynolide
desVIII/KTE	No macrolides produced	methymycin, neomethymycin
te/KTE	10-deoxymethynolide	Not determined
te+desVIII/KTE	methymycin, neomethymycin	methymycin, neomethymycin

strains using *Streptomyces-E. coli* shuttle vector pDHS702. The resulting strains, te/KTA and te+desVIII/KTE, were fermented under the 12-membered ring production conditions, and their macrolide products were analyzed by TLC. The results are summarized in the second column of Table 4-1. The expression of *te* in KTE-2 restores the production of aglycone, 10-deoxymethynolide (**65**), and the expression of both *te* and *desVIII* in KTE-2 re-establishes the biosynthesis of methymycin (**1**) and neomethymycin (**2**). These results demonstrated that, except for TE and DesVIII, all other components of the methymycin biosynthetic machinery are intact in the KTE-2 mutant.

4.3.6H Challenge to the "Linear Substrate Hypothesis": Feeding Experiments

The evidence contradicting the "Linear substrate hypothesis" came from feeding experiments. Three *S. venezuelae* strains, KTE-2, desVIII/KTE, and te+desVIII/KTE, were grown under the 12-membered ring production conditions,

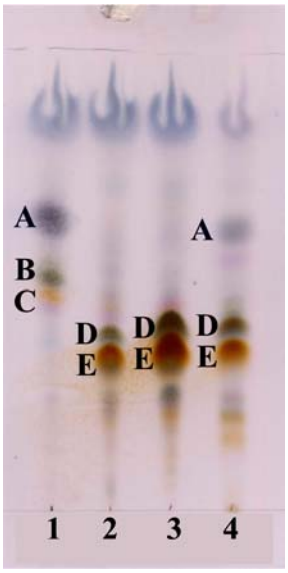

Figure 4-14: TLC analysis of macrolide production by *S. venezuelae* mutants fed with 10-deoxymethynolide. Strains are as follows: KTE-2 (lane 1), desVIII/KTE (2), te+desVIII/KTE (3), wild type (4).

then 10-deoxymethynolide (**65**) was added to the cultures and the growth was continued. A TLC analysis of the macrolides produced by these cultures revealed that (**65**) is taken up by these *S. venezuelae* cells and is modified by the enzymes of these strains. Figure 4-14 shows the TLC of the macrolide products and Table 4-1 (third column) provides a summary of the results. First, feeding of **65** (spot A) to KTE-2 led to its partial conversion to the hydroxylated aglycones, methynolide (**74**, B) and neomethynolide (**75**, C). This observation clearly indicated the cellular uptake of the aglycone and the subsequent modification by PikC. No glycosylated products were detected, since glycosylation apparatus was not restored. On the other hand, feeding of **65** (A) to desVIII/KTE and te+desVIII/KTE resulted in its conversion to the same compounds as produced by wild type, methymycin (**1**, D) and neomethymycin (**2**, E). The fact that in the

desVIII/KTE case where TE has been deleted the system is still capable of glycosylating **65**, strongly suggests that TE is not required for glycosylation when **65** is available to the cell. Therefore, the macrolactone, 10-deoxymethynolide (**65**), must be the substrate for the glycosyltransferase, DesVII. This result is inconsistent with the "Linear substrate hypothesis".

4.3.7 Optimization of DesVII Assay Conditions

As it became apparent that 10-deoxymethynolide (**65**) and TDP-D-desosamine (**22**) are indeed substrates for DesVII, we again turned our attention to search for the missing ingredients or special conditions for the glycosyl-transferase activity. The first test was to run the assay using a series of Tris buffers at different pH (5, 6, 7, 8, 9) with DesVII-C-His purified from *E. coli*. A TLC analysis revealed the formation of a new product in trace amount, as compared to the control without DesVII, at pH 8 and 9. This product has R_f value similar to that of YC-17 (**3**). The yield of this product, although significantly higher at pH 9 than 8, was very low and did not improve after extending the incubation time to 19 h. The amount of the product was not sufficient for any further analysis except by TLC.

Several changes to the original assay composition (Section 4.2.5) were also made in a hope of achieving better conversion. For instance, to improve the stability of the proteins, BSA was added to the assay mixture at 1 mg/mL. Since the enzyme activity is often inhibited by the presence of organic solvent, the concentration of 10-deoxymethynolide ethanol stock solution was increased from 20 to 50 or 100 mM so as to minimize the volume of ethanol stock necessary for

the assay mixture. Also, the concentration of 10-deoxymethynolide (**65**) in the assay mixture was raised from 0.5 to 1 mM to ensure the supply of the aglycone substrate. Meanwhile, the concentration of TDP-D-desosamine (**22**) was altered from 2 to 0.8 mM to prevent any substrate inhibition toward DesVII. Incubation time at 29 ^{o}C was also varied from hours to days to push for maximum conversion. While, based on the analysis of other glycosyltransferases, DesVII is not expected to require any cofactors, the addition of common cofactors, such as PLP, PMP, FMN, FAD, NADH, and NADPH, was nevertheless attempted. None of these changes resulted in a noticeable improvement of DesVII activity.

4.3.8 Involvement of DesVIII in Glycosyl Transfer by DesVII

To check whether DesVIII is needed for the glycosyltransferase activity of DesVII, a sample of DesVIII was needed. Dr. Lishan Zhao had tried to express *desVIII* in *E. coli* BL21 using a pET expression system, but no expression was detected. Attempt was also made to express *dnrQ* as a replacement for *desVIII*, since DnrQ can complement DesVIII activity *in vivo* (Chapter 2). Unfortunately, our efforts to produce DnrQ in *E. coli* as *C*- and *N*- terminal His_6 tag fusions resulted in low yields of proteins which failed to bind Ni-NTA agarose. Thus, the main focus of our research on this subject was directed to improve the heterologous expression of *desVIII* and to purify the corresponding protein by different methods. The results are described below.

4.3.8A Simultaneous Expression of pikAV and desVIII

It was found by Reynolds, Sherman and coworkers that the deletion of 687 bp of the 843 bp *pikAV* in *S. venezuelae* leads to a strain unable to glycosylate

polyketide aglycones.[82] The authors suggested that the deleted region contains a transcription unit essential for the expression of the *des* genes, *desVIII*, *desVII*, and *desVI*. Although it is likely that this unit may only be required for gene expression in *Streptomyces* strains, we decided, for the sake of precaution, to include *pikAV* in our expression system when express *desVIII* in *E. coli*. It should be noted that the *pikAV* and *desVIII* genes are translationally coupled, meaning that the stop codon of *pikAV*, TGA, overlaps with the start codon of *desVIII*, GTG.

Genes *pikAV* and *desVIII* were cloned as a single DNA fragment in the pET24b(+) vector, and the resulting plasmid, pikAV-desVIII/pET24b, was used for the expression in *E. coli* BL21. Two protein bands of approximately 32 and 43 kDa were clearly visible on SDS-PAGE of the isolated crude extracts, indicating the presence of PikAV and DesVIII-C-His (abbreviated DesVIII-1 later in text), respectively. However, DesVIII-1 failed to bind to the nickel affinity resin. It was determined later by Western blot analysis that DesVIII-1 protein does not contain a histidine tag. DNA sequencing confirmed that there is a stop codon immediately flanking the *C*-terminus of *desVIII,* possibly due to a frame-shift in the pET24b vector used in this cloning. A DEAE anion exchange chromatography was used to purify this protein. Thus, a crude but enriched DesVIII-1 sample was obtained that was used in the subsequent activity assays. Due to the presence of many contaminants, the concentration of DesVIII-1 in this sample was not determined.

4.3.8B DesVIII Significantly Improves the Yield in Glycosyltransferase Assay

The crude sample of DesVIII-1 was added as a new component to the optimized glycosyltransferase assay discussed above. The extent of the reaction was monitored by TLC. After 2 h incubation, a slight improvement in the YC-17 yield was noted in the reactions where DesVIII-1 was present as compared to the incubation without DesVIII-1. More significant improvement was found when the incubation was continued for 19 h. Under this condition, almost all of the substrate was converted to the product (>90% as estimated by TLC) in the assay mixture containing DesVIII-1, whereas only approximately 5% of 10-deoxymethynolide (**65**) was converted to YC-17 (**3**) in the absence of DesVIII-1. Further extension of incubation time did not visibly change the substrate/product ratio in either case. A preparative TLC was used to isolate the reaction product, which was verified to be YC-17 by mass spectroscopy using a chemical ionization technique. The structure of the product was later confirmed by NMR analysis of the product obtained from a large scale incubation. The ^{1}H and ^{13}C NMR data are in excellent agreement with published spectra, unequivocally proving it to be YC-17 (**3**).[42], [32]

The results of this assay have allowed us to define the necessary conditions for DesVII activity. Two important discoveries are: first, DesVIII must be included in the assay mixture in addition to DesVII, and second the reaction should be buffered to pH 9. However, a sample of pure DesVIII was needed to further investigate its involvement in the reaction.

4.3.8C Improvement of DesVIII Purification Method

Purification of DesVIII with C-terminal His_6 tag. Gene *desVIII* was cloned into pET24b(+) and the resulting clone, pDesVIII-C-His, was used to transform *E. coli* BL21. The protein, DesVIII-C-His (DesVIII-2, 43.6 kDa), was clearly over-produced by *E. coli* after the addition of IPTG, however, it was produced mainly as inclusion bodies. Some attempts were made to adjust the expression conditions in order to obtain a soluble DesVIII-2. These included using different *E. coli* colonies, different media (LB and 2×YT), different IPTG concentrations, different induction times and temperatures. None of these changes improved the protein solubility. The expression in *E. coli* NovaBlue strain did not produce any desired protein at all.

The insoluble DesVIII-2 protein pellet was collected and solubilized in a buffer containing 8 M urea. The denatured but soluble DesVIII-2 was subjected to nickel affinity purification. This method yielded an impure protein sample of DesVIII-2 (approximately 1 mg/mL of DesVIII-2, Figure 4-4, lane 2). The attempts to further purify this sample by FPLC with MonoQ or Superdex columns or concentrate it led to protein precipitation. When the DesVIII-2 sample was used in the glycosyltransferase activity assay it, like DesVIII-1, was effective to significantly increase the yield of the product, as compared to the controls in which DesVIII-2 was absent.

Purification of DesVIII with N-terminal His_6 tag. In order to generate an *N*-terminal His_6-tagged protein, *desVIII* was cloned into the pET28b(+) vector. The resulting plasmid, pDesVIII-N-His, was used to transform the expression host

E. coli BL21. To our delight, this strain over-produced DesVIII-N-His (DesVIII-3) in soluble form. Western blot analysis confirmed that DesVIII-3 contains a histidine tag. However, DesVIII-3 can not bind to the nickel affinity resin. Despite numerous attempts, we failed to purify DesVIII-3 using different affinity (cobalt-containing TALON), ion exchange (DEAE), or size exclusion (Sephacryl) resins.

The activity of DesVIII-3 was evaluated during the protein purification attempts. It dropped significantly after each step, possibly due to the prolonged incubation at 4 ^{o}C. The recovery of DesVIII-3 was also quite low after each column run, probably due to the protein precipitation upon loading onto the column.

Purification of DesVIII Fused to Maltose Binding Protein. A useful expression vector was developed by a member of our group, Peng Gao. Using this vector, MalE-pET, the protein of interest is expressed in fusion with maltose binding protein (MBP). The MalE-pET plasmid is derived from the pET vectors family and contains gene *malE,* coding for MBP, followed by the sequences for the His_{10} tag, Tev protease cleavage site, and the restriction endonuclease recognition sites. A gene cloned into the *Nde*I-*Hind*III sites of such a plasmid would be expressed in *E. coli* host having MBP and His_{10} tag at its *N*-terminus. MBP would direct the proper folding of the downstream protein, and the fusion can be purified by a nickel affinity chromatography. It can then be cleaved by Tev protease whose cleavage site, Glu-X-X-Tyr-X-Gln/Ser↓, is located after the His_{10} tag and right before the start of the protein of interest. The desired protein

can then be separated from MBP using a second affinity column. Among many advantages of this method is that the final protein will not contain any tags and will have a sequence identical to that of the native protein (with the exception of the first amino acid being valine instead of methionine in the case of DesVIII).

The *desVIII* gene was cloned into the MalE-pET vector, and the resulting construct, pDesVIII-MalEpET, was used for the expression in *E. coli* BL21. The fusion protein, MalE-DesVIII (88.9 kDa), was overproduced and present in the soluble fraction. It binds to Ni-NTA agarose well, and is a major component of the imidazole elution fractions although other protein impurities are also present. The fraction containing the partially purified fusion protein, together with DesVII, was found to be active in the glycosyltransferase assay. Upon digestion with Tev protease, the 88.9 kDa band disappeared, and two major bands, 42.2 kDa for DesVIII-4 and 46.7 kDa for MBP, were visible on SDS-PAGE. Purification by a second nickel affinity column resulted in the removal of most of MBP. The remaining trace of MBP was removed using an amylose affinity column.

We were able to concentrate DesVIII-4 to approximately 5.5 mg/mL before the formation of precipitate became visible. Despite the presence of minor impurities, this purification technique produces the best DesVIII sample in terms of purity (Figure 4-8). The DesVIII-4 protein is active in the glycosyltransferase assay. A preliminary estimation of the native molecular mass of DesVIII-4 by size exclusion chromatography gave a value of 57 kDa, which is consistent with a monomeric composition.

4.3.8D Substrate Preferences in DesVII/DesVIII-Catalyzed Reaction

Having obtained purified and active DesVII-C-His and DesVIII-4 proteins, we could now investigate the substrate preference of this catalytic pair. We tested a number of donor and acceptor substrates available to us. The reaction products in the incubation mixture were first detected by TLC and then purified by preparative TLC or HPLC followed by high-resolution mass spectroscopy analysis (CI, positive mode).

Two alternative HPLC solvent systems were used, both employing a reverse phase C_{18} analytical column. Traditionally, we eluted macrolide compounds using isocratic 30–70% acetonitrile in 0.057 M ammonium acetate. This solvent works well for aglycones or when the sugar moiety of the macrolide does not possess an amine functionality. However, we discovered that peaks become broad, and the retention time fluctuates for the macrolides containing amino sugars under the above elution conditions. It was later found that the solvent system developed for the separation of tylosin derivatives works better.[43] In this method, compounds are eluted with a gradient of acetonitrile in 0.014 M triethylamine, with its pH adjusted to 3 with trifluoroacetic acid. The compounds were analyzed by TLC or MS after the removal of the solvent.

Figure 4-15 depicts the aglycone and sugar nucleotide substrates tested in the assay with DesVII/DesVIII catalytic pair. The isolated hybrid products are also shown. Figure 4-16 shows HPLC traces for the assay mixtures with the corresponding peaks labeled. It should be pointed out that the reactions shown in Figure 4-16 were conducted in the presence of excess aglycone substrate to

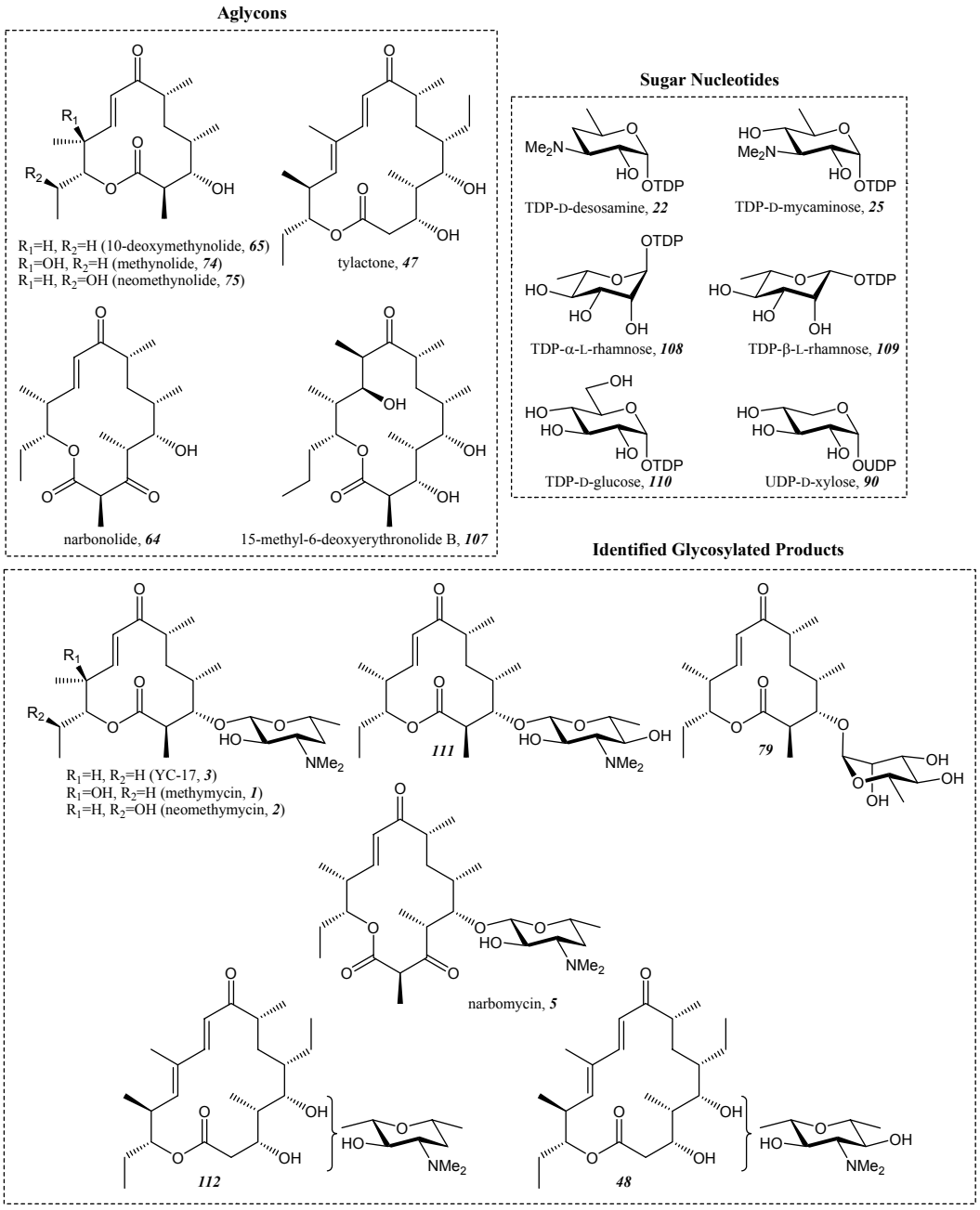

Figure 4-15: Macrolide aglycones and sugar nucleotides tested as substrates in the DesVII/DesVIII assay. The products of reactions are also shown.

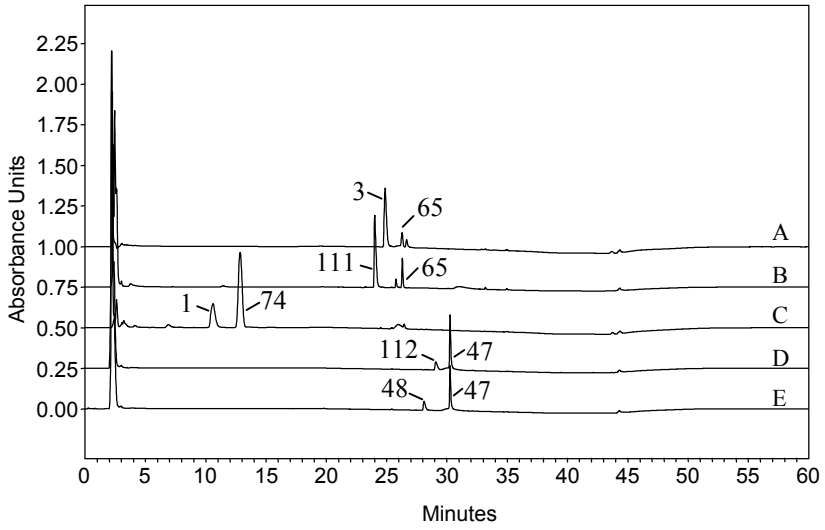

Figure 4-16: Representative HPLC traces for the reactions catalyzed by DesVII/DesVIII with the following substrates: A. 10-deoxymethynolide (**65**) and TDP-D-desosamine (**22**), B. **65** and TDP-D-mycaminose (**25**), C. methynolide (**74**) and **22**, D. tylactone (**47**) and **22**, E. **47** and **25**.

illustrate the differences in retention times of substrate and product. Complete consumption of the aglycone was observed in most cases when sufficient amount of donor sugar nucleotide was added. Table 4-2 provides a summary of all the substrates tested and the high resolution MS and HPLC data of the isolated products.

As expected, DesVII/DesVIII pair was capable of adding desosamine onto natural acceptors, 10-deoxymethynolide (**65**) and narbonolide (**64**) to yield natural products **3** and **5**, respectively. It was also able to desosaminylate hydroxylated aglycones **74** and **75** to yield methymycin (**1**) and neomethymycin (**2**), respectively. These observations are interesting, since it has been established that in *S. venezuelae* glycosylation precedes hydroxylation in the final tailoring steps

Table 4-2: Summary of Glycosylation Reactions[a]

		Aglycone Acceptors					
		65	**74**	**75**	**64**	**47**	**107**
Sugar Nucleotide Donors	**22**	**3** 25.0 min[b] 454.3172[c] 454.3169[d]	**1** 10.6 min 470.3123 470.3118	**2** 10.1 min 470.3123 470.3118	**5** 26.6 min 510.3418 510.3431	**112** 29.1 min 552.3867 552.3900	NP[e]
	25	**111** 24.0 min 470.3117 470.3118	ND[f]	ND	ND	**48** 28.1 min 568.3832 568.3849	ND
	108	NP	ND	ND	ND	NP	ND
	109	**79** 23.0 min 443.2630 443.2645	ND	ND	ND	NP	NP
	110	NP	ND	ND	ND	NP	ND
	90	NP	ND	ND	ND	ND	ND

[a] Substrates and products are numbered as in Figure 4-15. [b] HPLC retention time (HPLC conditions are described in Section 4.2.22). [c] Results of a high-resolution CI^+ MS. [d] Calculated [M+H] values. [e] No product detected. [f] Not determined.

(Section 1.5). Therefore, our results indicate that DesVII/DesVIII pair is fairly flexible with respect to the side chains of the aglycone substrate.

We were unable to detect any product when 15-methyl-6-deoxy-erythronolide B (**107**), a methylated analog of erythromycin precursor 6-deoxy-

erythronolide B (**15**, Figure 1-10), was used as the acceptor substrate. Just like narbonolide (**64**), this compound is a 14-membered macrolactone, but differs from **64** by having a hydroxyl group at C-3, instead of a keto group, additional methyl groups at C-10 and C-15, and the absence of a double bond at C_{10}-C_{11}. Any of these structural variations may have contributed to the lack of recognition in the glycosyl transfer reaction.

Because tylactone (**47**) contains two potential glycosylation sites, 3-OH and 5-OH, two different singly glycosylated products could be generated in our experiment. Since mass spectroscopy was used to determine the chemical nature of the products, it is impossible to distinguish whether **112** and **48** are glycosylated at 3-OH or 5-OH positions. However, it is clear that only one sugar is attached in both cases, and a single product is generated in each reaction. It should be pointed out that the natural product, tylosin (**13**), made by *S. fradiae*, has a mycaminose attached at 5-OH position of tylactone, and **48** is likely an intermediate in the tylosin biosynthetic pathway (Figure 1-11). Compound **112** was previously reported by Gaisser and coworkers.[83] In their study, the authors used a mutant of *S. erythraea*, erythromycin producer, capable of biosynthesizing TDP-D-desosamine and TDP-L-mycarose but lacking the necessary glycosyl-transferases for their attachment and the entire polyketide synthase machinery. To this mutant was introduced a plasmid carrying the gene encoding the glycosyl-transferase of tylosin biosynthesis, TylM2, (Fig. 1-11). After feeding the mutant strain with tylactone, 5-desosaminyl-tylactone (**112**, desosaminylated at 5-OH) was detected in the fermentation broth, and its structure was confirmed by NMR

spectroscopy.[83] Taking into consideration the results obtained by Gaisser and coworkers[83] and the fact that narbonolide is also desosaminylated at 5-OH, it is likely that our product is also the 5-desosaminyl-tylactone (**112**).

We also found that DesVII/DesVIII is able to utilize TDP-D-mycaminose (**25**) with approximately the same efficiency as its natural substrate, TDP-D-desosamine (**22**). This finding is not surprising since these two compounds only differ in the presence of an additional C-4 hydroxyl group in mycaminose. Our previous *in vivo* experiments have already demonstrated *S. venezuelae*'s ability to accept various biosynthetic intermediates of desosamine, including the ones substituted at C-4: D-quinovose[29] and 4-amino-4,6-dideoxy-D-glucose.[68]

Only TDP-β-L-rhamnose (**109**) and not TDP-α-L-rhamnose (**108**) is a substrate for DesVII/DesVIII, and the yield of the glycosylated product **79** is significantly lower than those of the desosaminylated and the mycaminosylated compounds. The *in vivo* incorporation of L-rhamnose into macrolides in *S. venezuelae* mutant has been reported by our group.[84]

The fact that TDP-D-glucose (**110**) is not a substrate in our assay is not a total surprise. This compound is an important precursor in many primary and secondary metabolite pathways and is abundant in the cell. The fact that it has never been detected as a part of a naturally occurring macrolide structure indicates that it is not a substrate for this type of glycosyltransferases. To our best knowledge, there is only one example in which glucose is attached to a macrolide moiety. The discovery was made by Gaisser and coworkers, where mutant *S. erythraea* deficient in macrolide glycosyltransferases was able to glucosylate

exogenous tylactone to produce 5-*O*-glucosyl-tylactone.[83] However, the identity of the responsible glycosyltransferase remains unknown. In contrast, glucose is a common donor substrate for the glycosyltransferases catalyzing glycosylation of macrolide antibiotics as a part of the organism's resistance mechanism (Chapter 3). However, these glycosyltransferases catalyze bond formation between two glycosyl moieties rather than between glucose and the macrolide acceptor.

Because D-quinovose is a substrate of DesVII, we expected that UDP-D-xylose (**90**) may be recognized by DesVII/DesVIII as well. However, this was not the case. It is possible that DesVII/DesVIII has a strict preference for TDP over UDP nucleotide. But it cannot be excluded that the absence of the methyl group at C-5 of xylose may also be a determining factor.

Several of the compounds produced in this study, including **79**, **111**, and **112**, have not yet been found as natural products. Out of these, **111** is a novel compound, which has never been reported. The results presented here demonstrate that DesVII/DesVIII pair can catalyze coupling of various aglycone and sugar derivatives that are not their natural substrates. Due to the limitation in the selection of both acceptor and donor substrates, no correlations can be made at this point to define the structural elements of the substrates necessary for recognition by the catalyst(s). Nevertheless, the development of an *in vitro* system greatly simplifies such structure activity relationship studies. More importantly, the data collected in the presented work, combined with the previous

in vivo studies have clearly established the feasibility of glycodiversification of macrolides by using the glycosyltransferase systems.

4.3.8E The Place of DesVIII Among Its Homologues

The BLAST search of the protein database led to a number of DesVIII homologues in bacterial genomes. On the top of the list are thirteen proteins (at the time of writing, including DesVIII) whose catalytic functions are associated with the biosynthesis of glycosylated secondary metabolites in bacteria, for the most part streptomycetes. The majority of them have been assigned as tautomerases or 3,4-isomerases, just as DesVIII was speculated initially. Generally, they are charged with the biosynthesis of a specific sugar unit in each respective pathway. They all have an end-to-end similarity to P450 hydroxylases but lack the P450 signature motif, including the heme-ligating invariant cysteine residue.

These DesVIII homologues are followed by a large number of authentic bacterial P450 enzymes which have a typical P450 signature motif and an

```
                    390                 ↓        420
 P450, B. subtilis  TRSP---NPHLSFGHGHHVCLGSSLARLEAQ
 P450, M. ulcerans  TRANA--NRHLAFSGGRHFCLGAALARVEGE
P450, G. violaceus  TRVD---NRHLAFGDGIHHCFGGPLARVEGQ
 P450, C. watsonii  QRS----NRNLPFGGGIHFCLGAFLARLQGQ
   P450, M. leprae  DRPS---SRHLAFAVGSHFCLGAALARLEAT
DesVIII, S. venezu  ARPDA--AAHLALHPAGPYGPVASLVRLQAE
DnrQ, S. peucetius  DRPRSPGFTHMALAGRDHLGLVAPLVRVQCT
EryCII, S. erythra  DRPD---ADRALSAHRGHPGRLEELVTALAT
         Consensus   R     N HLAFG G H CLGASLARLEA
```

Figure 4-17: Alignment of selected DesVIII homologues and bacterial cytochrome P450 enzymes. Shown in bold is the bacterial P450 signature motif: F G X G X H/R X C L G/A with the heme-ligating cysteine marked with ↓. These features are absent in DesVIII, DnrQ, and EryCII.

invariant cysteine residue. Protein sequence alignment of these P450 enzymes (only those more similar to DesVIII were selected) with DesVIII and its "tautomerase" homologues (Figure 4-17) shows that, despite an overall 30–40% sequence identity with DesVIII, these two groups of enzymes are clearly different. Figure 4-18 depicts a phylogenetic tree illustrating the degree of sequence similarity between DesVIII and its closest homologues among "tautomerases" and P450s. It is apparent that the degree of sequence homology between different DesVIII homologues shows correlation with the structure of the acceptor substrate being glycosylated, whether it is a macrolide or an aromatic polyketide, and whether it has sugar moieties attached to it already. This is consistent with the fact that DesVIII, and most likely its homologues, are involved in glycosyl transfer rather than the biosynthesis of the unusual sugar moieties.

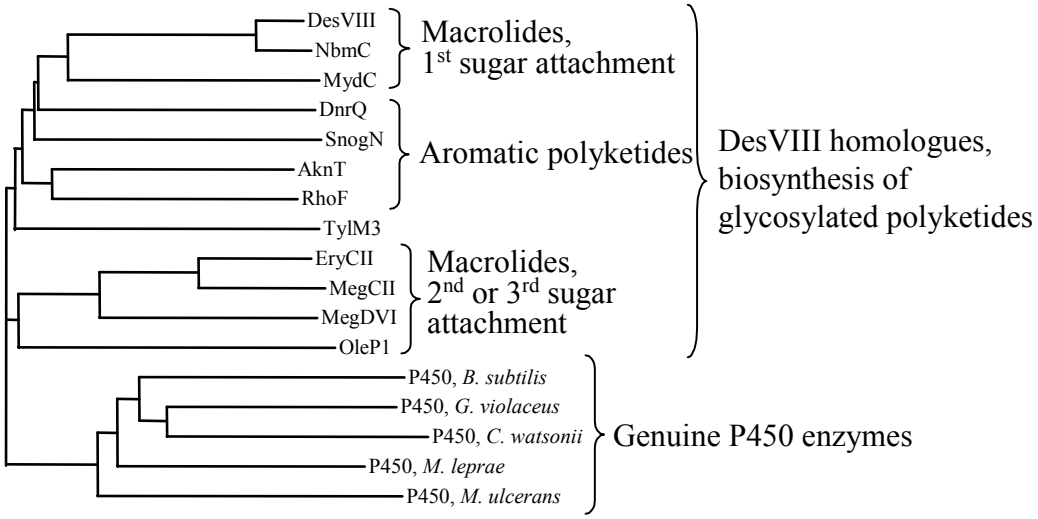

Figure 4-18: Phylogenetic tree of the closest DesVIII homologues.

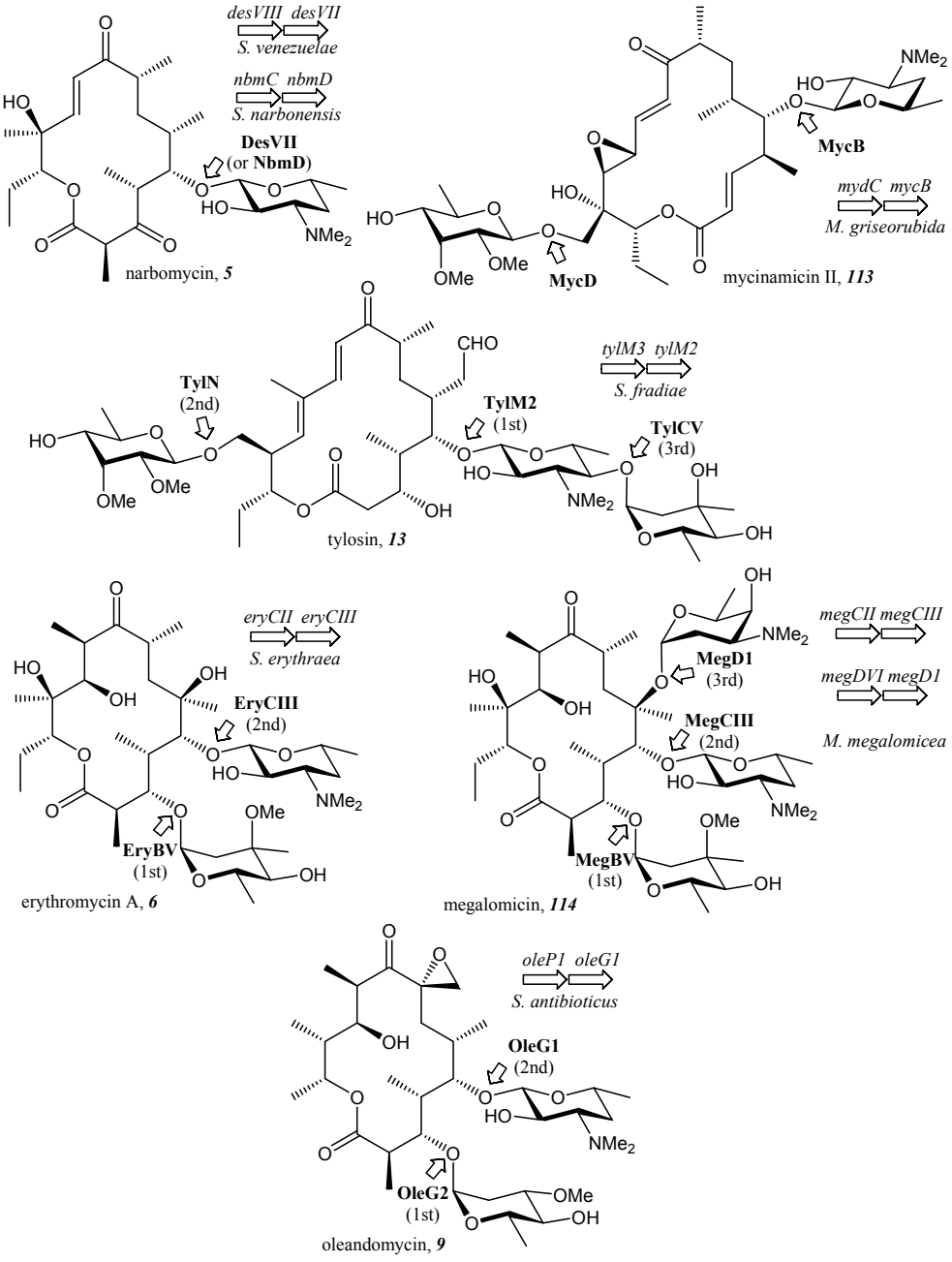

Figure 4-19 A: Structures of the macrolide compounds discussed in the text and the relevant portions of their biosynthetic gene clusters.

There is little biochemical data available on the specific function of any of the DesVIII homologues involved in the biosynthesis of glycosylated polyketides. Their functions have been tentatively assigned based only on gene knockout experiments and/or sequence comparison. Figure 4-19 provides the structures of the representative polyketides and the corresponding putative glycosyltransferases for each glycosylation step. The order of glycosylations in each compound is also shown, when known.

The closest DesVIII homologue, NbmC (70% identity), is a part of narbomycin (**5**) biosynthesis in *Streptomyces narbonensis* and presumably has the same function as DesVIII.[43] Genes encoding MydC (42% identity to DesVIII) and TylM3 (34%) are found in the biosynthetic clusters of mycinamicin II (**113**, *Micromonospora griseorubida*)[85] and tylosin (**13**, *S. fradiae*),[46] respectively, and are involved in the biosynthesis or attachment of the first sugar moiety to the aglycone. OleP1 (30%) of oleandomycin (**9**, *S. antibioticus*),[45] EryCII (31%) of erythromycin (**6**, *S. erythraea*),[47] and MegCII (29%) as well as MegDVI (35%) of megalomicin (**114**, *Micromonospora megalomicea*)[86] biosynthetic pathways are involved in the attachment of sugar moieties to already glycosylated macrolides (Figure 4-19 A). DnrQ (35%), SnogN (33%), AknT (33%), and RhoF (33%) are involved in the glycosylation of aromatic polyketides doxorubicin (**115**, *S. peucetius*),[87] nogalamycin (**116**, *Streptomyces nogalater*),[88] aclarubicin (**117**, *Streptomyces galilaeus*),[89] and β-rhodomycin II (**118**, *Streptomyces violaceus*),[90] respectively (Figure 4-19 B).

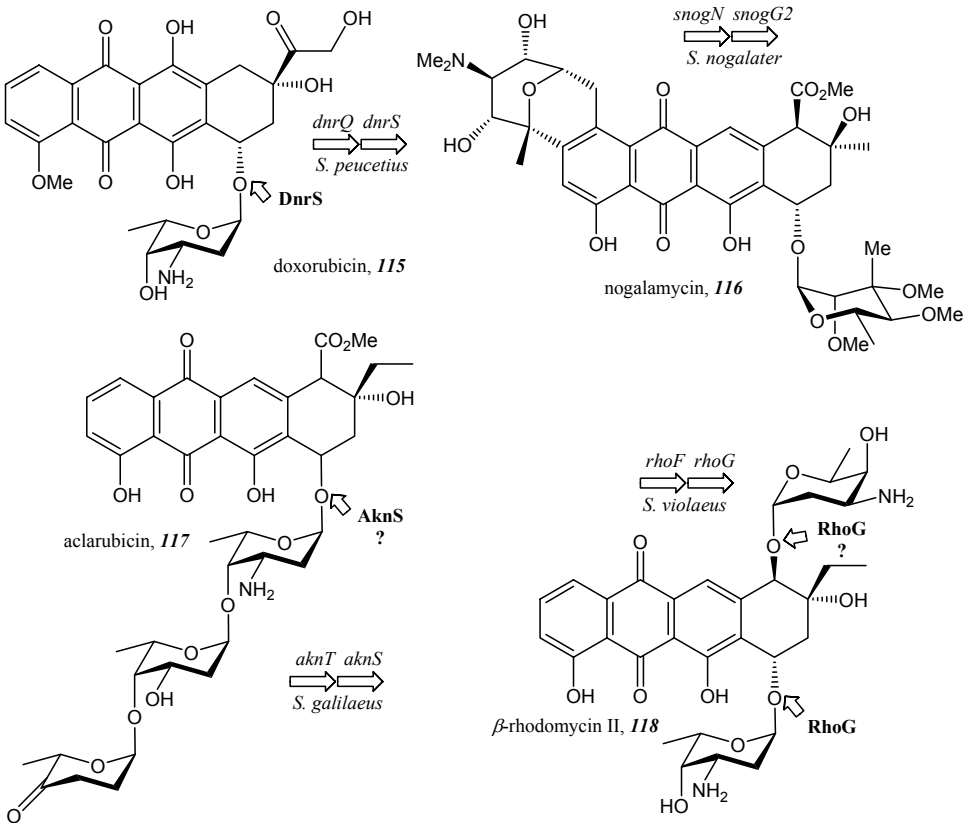

Figure 4-19 B: Structures of the aromatic polyketides discussed in the text and the relevant portions of their biosynthetic gene clusters.

Interestingly, in twelve of the thirteen cases these glycosylated metabolites contain an amino sugar and the DesVIII homologues are likely involved in the biosynthesis or attachment of the amino sugar in the respective pathways. The exception is the DesVIII homologue encoded by *lkm32* from the biosynthetic gene cluster of lankamycin in *Streptomyces rochei*.[91] Lankamycin (not shown) does not contain an amino sugar moiety. In this case, however, the identified gene cluster is not a part of the chromosome but part of a plasmid, which could be transferred as an incomplete portion from some other *Streptomyces* species.

Another interesting feature of the pathways including a DesVIII homologue is the location of this homologous gene in each gene cluster. The sugar biosynthetic genes found in the gene clusters of polyketide secondary metabolites are generally clustered, but their relative arrangement can be different from case to case. The genes homologous to *desVIII*, however, are almost always followed by a glycosyltransferase gene (Figures 4-19 A and B), and each pair is involved in the biosynthesis or attachment of the same sugar moiety. Out of the thirteen cases discussed here, the only exception is the nogalamycin gene cluster of *S. nogalater*, where *desVIII* homologue, *snogN*, is followed by *snogG2* encoding a putative *C*-methyltransferase.[88] This, however, may be related to the special mode of attachment of an amino sugar moiety to an aromatic aglycone, in which formation of a C-C bond occurs in addition to the glycosidic coupling.

Overall, the consecutive arrangement of *desVIII* and *desVII*, and in many other cases, can be considered a general trend. Recognition of this pattern is useful in the assignment of gene functions in other biosynthetic clusters or in the prediction of the structures of unidentified secondary metabolites.

Because of the likely connection between the presence of *desVIII* homologues in the biosynthetic gene clusters and amino sugars in the produced compounds, it is tempting to assume that DesVIII homologues are specifically required for the transfer of amino sugars. However, our *in vitro* experiments demonstrate that DesVII/DesVIII pair can also catalyze the attachment of L-rhamnose, and the glycosyl transfer is undetectable in the absence of DesVIII. It is conceivable that DesVIII and its homologues are coevolved with the

corresponding "DesVII" to catalyze the amino sugar transfer. This protein pair works synergistically even when a neutral sugar is used as an alternative substrate.

However, there exist some glycosyltransferases involved in the attachment of amino sugars in the biosynthesis of secondary metabolites that are not accompanied by a DesVIII equivalent. These include GtfC and GtfD participating in the biosynthesis of vancomycin and teicoplanin, respectively, both of which are nonribosomal derived peptide antibiotics.[9, 10] Likewise, FscMI, the glycosyltransferase involved in polyene macrolide antibiotic FR-008 biosynthesis also lacks a DesVIII equivalent.[92] These exceptions may be due to the different mechanisms of glycosylation and/or the differences in the structures of aglycones.

4.3.8F Role of DesVIII in Glycosylation Reaction: Initial Studies

It is evident from the results discussed above that the glycosyl transfer reaction requires DesVII, and the presence of DesVIII substantially increases the glycosyl transfer efficiency. However, DesVIII is not absolutely required for glycosyl transfer, since low conversion of glycosylation is still possible in its absence under alkaline conditions. Because DesVII performs the actual chemical reaction of glycosyl transfer in methymycin/pikromycin biosynthesis, the role of DesVIII in this process, although identified as being important, is not immediately apparent. Our attempts to investigate its function were hampered by the difficulties encountered in obtaining pure DesVIII sample. Therefore, only preliminary results and qualitative trends of its characterization are reported here.

Probing Protein-Protein Interactions between DesVII and DesVIII. In our search of appropriate assay conditions, we observed that increasing the amount of DesVIII with the constant amount of DesVII resulted in the increase in the % conversion (See Figure 4-21 for an example showing such a trend). In order to determine an optimal molar ratio of DesVII to DesVIII it is important to establish that there is an interaction between DesVII and DesVIII. Two different methods were used to probe protein/protein interactions.

Size exclusion chromatography was performed on individual DesVII and DesVIII proteins, and the DesVII/DesVIII mixture. The FPLC trace of DesVII/DesVIII mixture is practically superimposed to the individual traces of DesVII and DesVIII, and no protein peak with a molecular mass corresponding to the sum of these two proteins (at least 90 kDa, depending on subunits' ratio and number) was detected. However, the results were complicated by the fact that significant amounts of DesVIII was in the aggregated state. Therefore, even though there exists no obvious interaction between DesVII and DesVIII proteins, the results are not conclusive.

The Far Western blot analysis is another method employed to probe the DesVII/DesVIII interaction. In this experiment, untagged DesVIII-1 was first separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was then exposed to the solution containing DesVII-C-His, washed, and probed with an anti-histidine antibody. The detection of a chemiluminescent band on the membrane would indicate the presence of a ternary complex between antibody, DesVII-C-His, and the immobilized DesVIII-1. Unfortunately, despite

many attempts, no such band was detected. Therefore, this experiment also failed to show the interaction between DesVII and DesVIII. It is possible that transfer of DesVIII to the membrane in the presence of methanol irreversibly changed its conformation or the conformation of the crucial recognition motifs. It is also possible that during the transfer, blocking or the washing steps, DesVIII simply denatures, since, as it has been before, DesVIII tends to form inclusion bodies during purification. Therefore, this method might also be unreliable in determining protein interaction in our system.

The data obtained here do not support the protein-protein interaction hypothesis between DesVII and DesVIII. However, none of the methods used was completely reliable. It should be mentioned that using a yeast two-hybrid system, a member of our group, Allen Yu, has obtained preliminary results indicating the existence of interaction between DesVII and DesVIII *in vivo*.

Speculation of the Roles of DesVIII in Glycosyl Transfer.

Hypothesis 1: DesVIII Serves as DesVII-reactivating Enzyme. Preliminary observation showed that when nearly full consumption of the aglycone was achieved in the presence of DesVIII and sufficient amount of sugar substrate; the control reaction without DesVIII only resulted in approximately 5% conversion of aglycone into a glycosylated product. Prolonged incubation did not improve the yield of the product in the absence of DesVIII. We estimated that the amount of the product generated in the absence of DesVIII is roughly twice that of the amount of DesVII (two equivalents per DesVII monomer). It appears that only single turnover happens when DesVII acts alone. It is therefore possible that

DesVII is inactivated after each turnover and needs to be re-activated by an additional component, DesVIII.

On the basis of the protein sequence analysis and the anomeric configuration of sugar donors and glycosylated products, DesVII is clearly an inverting glycosyltransferase. Figure 4-20 A shows the established mechanism for this group of enzymes. Similar to other glycosyltransferases of the GT-B superfamily, DesVII does not possess a DXD metal binding motif found in members of the GT-A superfamily. In the enzymes of the GT-A superfamily this motif is used to coordinate a divalent metal, usually magnesium, which in turns stabilizes the leaving diphosphate group of the nucleotide.

We speculate that in DesVII reaction, perhaps the leaving nucleoside diphosphate, upon its displacement by a hydroxyl group of the acceptor substrate, is transferred to an acceptor residue of DesVII (Figure 4-20 B). It may remain bound to DesVII, covalently or not, and occupy the active site until removed by DesVIII.

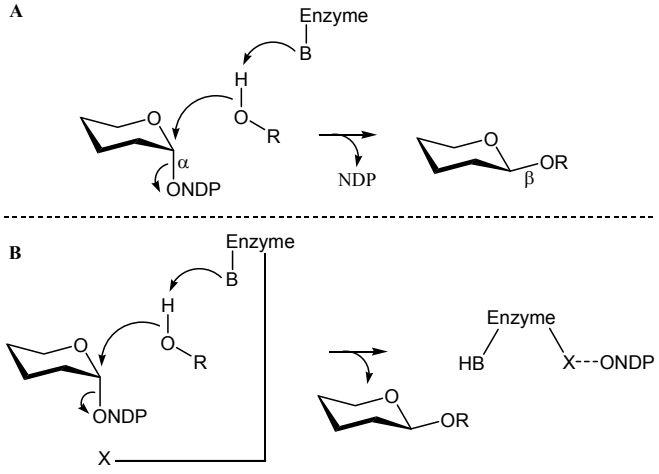

Figure 4-20: Mechanisms for inverting glycosyltransferases: (A) generally accepted mechanism; (B) proposed mechanism for DesVII reaction.

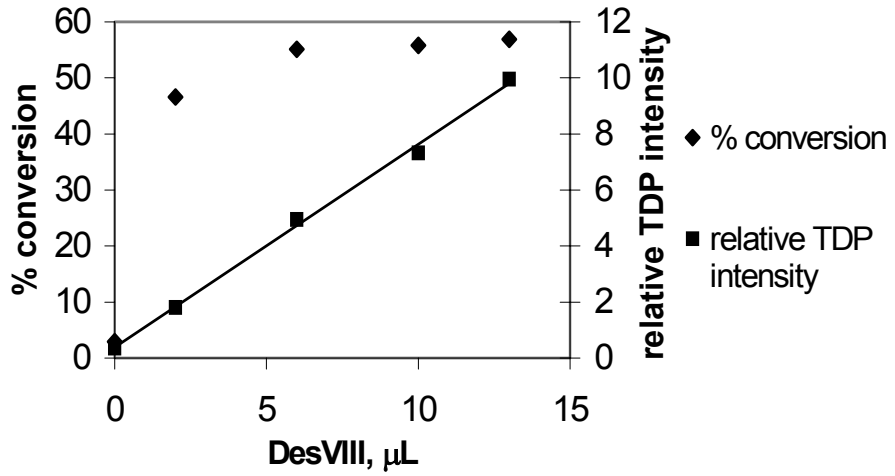

Figure 4-21: The dependence of % conversion (diamonds) and release of TDP (squares) on the amount of DesVIII in the DesVII/DesVIII assay. Release of TDP is estimated from the HPLC chromatogram as the ratio of the integration of peaks at 2.5 min to the sum of the peaks corresponding to **65** and **3**. The DesVII concentration is constant.

Support for this hypothesis comes from an interesting observation that the intensity of the TDP peak (retention time approximately 2.5 min, Figure 4-16) in HPLC trace of the DesVII/DesVIII reaction is higher when the amount of DesVIII is increased. Preliminary data illustrating the dependence of the intensity of the TDP peak in an HPLC chromatogram and the extent of conversion (% conversion calculated from the integrations of the substrate (**65**) and product (**3**) peaks on the HPLC chromatogram) on the amount of DesVIII is shown in Figure 4-21. In the absence of DesVII, the dependence of the TDP peak intensity on the amount of DesVIII exists as well (not shown). These finding may indicate that DesVIII is somehow involved in the release of TDP from DesVII or directly from the donor substrate (**22**) itself.

It should be noted that the DesVIII sequence does not contain a putative nucleotide binding domain, at least not a known one. Thus, this proposal, although plausible, is largely speculative. Additional information is definitely needed to determine its merit.

Hypothesis 2: DesVIII Protects Amino Group of Sugar Moiety from Oxidation by P450 Enzymes. It has been shown that the dimethylamino group of an unusual sugar in macrolide antibiotics, *e.g.* desosamine in erythromycin (R-NMe_2, where R is the remainder of sugar appendage), undergoes oxidation by P450 enzymes during drug metabolism in patient's body (mainly in liver).[1] The resulting nitrososugar (RNO) is a reactive species and binds strongly to the heme group of P450, forming an inactive complex. This leads to interference with the metabolism of other drugs by hepatic P450s.[93]

We speculate that DesVIII has evolved from P450 enzymes and has inherited their affinity to amino sugars of polyketides but lost the catalytic activity as a monooxygenase. Therefore, it can bind amino sugars, perhaps either as a free compound or as a part of an antibiotic, and protects them from oxidation by functional P450s of the bacterium until the biosynthetic product is excreted from the cell. It has been reported that hepatic P450 prefers to bind an unprotonated dimethylamino group,[94] and this is consistent with our observation of DesVII/DesVIII activity at alkaline pH.

To probe this hypothesis, we decided to analyze the effect of P450 enzymes on the DesVII/DesVIII activity. A microsome preparation of human hepatic cytochrome P450 CYP3A4 isozyme was selected for the study. This

isoform has a high affinity for macrolide antibiotics but can also oxidize other xenobiotics, such as testosteron and nifedipine. Most membrane-bound enzymes, including cytochrome P450, are associated with microsomes. The microsome preparations used in this study are derived from insect cells and also contained rabbit cytochrome P450 reductase. Generally, in order to observe cytochrome P450 monooxygenase activity, an NADPH or an NADPH generating system has to be added to the assay. However, since the binding property, not the monooxygenase activity, of P450 is probed in our study, no NADPH or NADPH generating system is needed.

In our experiments, microsomes were added to the DesVII/VIII assay as described in Section 4.2.17. Due to the low abundance of the P450 enzyme in the microsome preparation, the concentration of P450 in the assay (0.13 μM) was lower than those of DesVII (10.5 μM) and DesVIII (7.8 μM), although the microsome portion constituted 17% of the assay volume. The preliminary data showed some improvement in the degree of conversion from **65** to **3** (Table 4-3). This observed improvement was marginal in the presence of DesVIII but nearly doubled in the absence of DesVIII.

Table 4-3: The Effect of Cytochrome P450 on Glycosylation[a]

Entry	DesVIII-4	P450	% Conversion
1	+	–	93
2	+	+	99
3	–	–	4
4	–	+	10

[a] The assays were run as described in Section 4.2.17 and analyzed by HPLC. DesVII was present in all reactions.

In order to increase the amount of P450 in the assay, in a separate experiment, a purified human recombinant cytochrome P450 CYP3A4 enzyme was used (2.5 μM, concentrations of DesVII and DesVIII were as above). Unfortunately, there was no effect on the glycosyl transfer by this preparation of cytochrome P450. Clearly, a more thorough examination of the effects of the two preparations of cytochrome P450 on the glycosylation reaction is necessary. It is possible that variations of components other than cytochrome P450 in the microsomal preparation are affecting the results, most likely the components of the phospholipid membranes. Preparations of P450 from other sources should also be tried.

Hypothesis 3: DesVIII, as P450 Enzymes, Is Membrane Associated. Another explanation for the synergistic effect of DesVIII on DesVII activity, based on the sequence homology between DesVIII and P450 enzymes, is their shared affinity to membranes. The results of the experiments described above suggest that phospholipids in microsomal preparations may be responsible for increasing the efficiency of the glycosyl transfer. Aglycones are poorly soluble in water and likely bind to lipid membrane rather than circulate freely in the cytoplasm of the cell. It is possible that DesVIII brings aglycone substrate, DesVII, and sugar donor together to facilitate the glycosyl transfer. This hypothesis could also explain the tendency of DesVIII to aggregate during protein purification. Although DesVIII does not have an obvious membrane binding domain, it may have a hydrophobic surface in its three-dimensional structure. To probe this hypothesis more experiments must be conducted. These should include

the effects of phospholipids and detergents on the glycosylation by DesVII alone and in the presence of DesVIII.

4.4 CONCLUSIONS

This chapter describes the investigation into the glycosylation reaction in the biosynthesis of macrolide antibiotics by *S. venezuelae*. This study resulted in the purification of a recombinant glycosyltransferase DesVII, the development of the optimal assay conditions for the glycosyl transfer reaction, and the production of a few new hybrid macrolides carrying unnatural sugars. The most significant discovery is the necessary requirement of an additional protein component, DesVIII, in the glycosyl transfer reactions. The role of DesVIII is still elusive and several plausible hypotheses are suggested here. Further exploration is necessary to substantiate or debunk these hypotheses. The substrate specificity of DesVII/DesVIII pair was also studied.

This study demonstrated, for the first time, the *in vitro* activity of a glycosyltransferase involved in the biosynthesis of macrolide antibiotics.[14] The discovery of the involvement of DesVIII in the catalysis provided evidence indicating the DesVII/DesVIII pair as a unique glycosylation system not reported before. The presence of homologous *desVII*/*desVIII* gene pairs in other polyketide biosynthetic clusters points toward a more general existence of such a system. This information is essential for future applications of glycodiversification of secondary metabolites in a combinatorial approach.

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List of Abbreviations

ABC	ATP-binding cassette
ACP domain	Acyl carrier protein domain
Apr^R	Apramycin resistant
Apr^S	Apramycin sensitive
ArCP domain	Aryl carrier protein domain
AT domain	Acyl transferase domain
ATCC	American Type Culture Collection
BCIP	5-Bromo-4-chloro-3-indolyl-phosphate
BSA	Bovine serum albumin
C domain	Condensation domain
cDNA	Complementary deoxyribonucleic acid
CDP	Cytidine diphosphate
CI-MS	Chemical ionization mass spectrometry
CoA	Coenzyme A
DEBS	6-Deoxyerythronolide B synthase
DH domain	Dehydratase domain
DIG	Digoxigenin
DMSO	Dimethylsulfoxide

DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
D. radiodurans	*Deinococcus radiodurans*
DTT	Dithiothreitol
E. coli	*Escherichia coli*
ER domain	Enoyl reductase domain
Erm	Erythromycin resistance methylase
FAB-MS	Fast-atom bombardment mass spectrometry
$FADH^{-}$	Flavin adenine dinucleotide, reduced form
FPLC	Fast protein liquid chromatography
GFP	Green fluorescent protein
H. marismortui	*Haloarcula marismortui*
HPLC	High performance liquid chromatography
HR	High resolution
HRP	Horseradish peroxidase
IPTG	Isopropyl-β-*D*-thiogalactoside
Kan^{R}	Kanamycin resistant
KR domain	*β*-ketoreductase domain
KS domain	Ketosynthase domain
LB	Luria Bertani broth
MBP	Maltose binding protein

MFS	Major facilitator superfamily
MGT	Macrolide glycosyltransferase
MLS_B resistance	Macrolide, lincosamide and streptogramin B resistance
MOPS	4-Morpholinepropanesulfonic acid
MPLC	Medium pressure liquid chromatography
mRNA	Messenger ribonucleic acid
NAD^+	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced
NBT	Nitro blue tetrazolium
NDP	Nucleoside diphosphate
NMR	Nuclear magnetic resonance
NRPS	Nonribosomal peptide synthetase
OD_{600}	Optical density at 600 nm
ORF	Open reading frame
PCP domain	Peptidyl carrier protein domain
PCR	Polymerase chain reaction
PIKS	Pikromycin polyketide synthase
PKS	Polyketide synthase
PKS I	Polyketide synthase type I
PLP	Pyridoxal 5'-phosphate

PMP	Pyridoxamine 5'-phosphate
PMSF	Phenylmethylsulfonyl fluoride
Ppant	Phosphopantetheine
PPTases	Phosphopantetheinyl transferases
RBS	Ribosome binding site
RNA	Ribonucleic acid
RNase A	Ribonuclease A
rRNA	Ribosomal ribonucleic acid
RTS	Rapid Translation System
SAM	*S*-Adenosylmethionine
S. antibioticus	*Streptomyces antibioticus*
S. aureus	*Staphylococcus aureus*
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
S. erythraea	*Saccharopolyspora erythraea*
S. fradiae	*Streptomyces fradiae*
S. lividans	*Streptomyces lividans*
S. peucetius	*Streptomyces peucetius*
S. pneumoniae	*Streptococcus pneumoniae*
S. venezuelae	*Streptomyces venezuelae*

TCA	Trichloroacetic acid
TDP	Thymidine diphosphate
TE domain	Thioesterase domain
TES	*N*-Tris[hydroxymethyl]methyl-2-amino-ethanesulfonic acid
TFA	Trifluoroacetic acid
$Thio^R$	Thiostrepton resistant
TLC	Thin-layer chromatography
TSB	Tryptic soy broth
TTP	Thymidine triphosphate
UDP	Uridine diphosphate
UV-vis	Ultraviolet-visble spectroscopy

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